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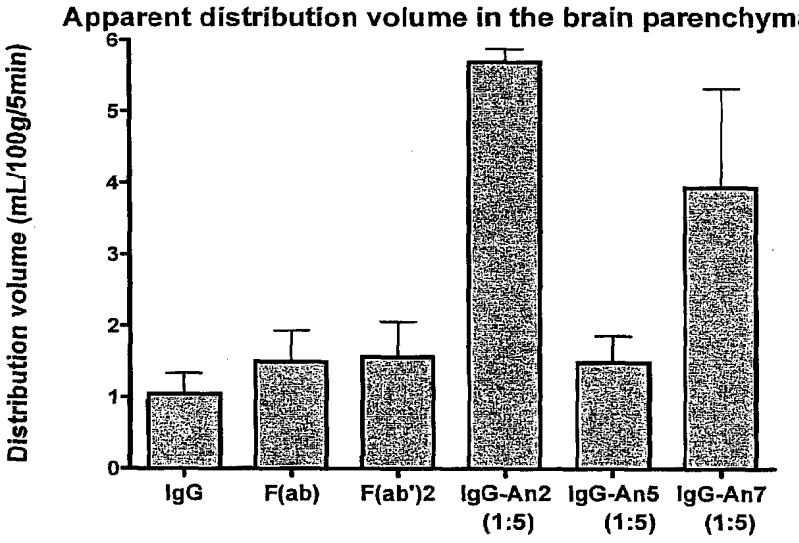

Fig. 16

(57) Abstract: Based on our identification of a polypeptide (Angiopep-7) that is efficiently transported to cells such as liver, lung, kidney, spleen, and muscle, the invention provides polypeptides, conjugates including the polypeptides, and methods for treating diseases associated with these cell types. Unlike other aprotinin related polypeptides identified herein (including Angiopep-3, Angiopep-4a Angiopep-4b Angiopep-5, and Angiopep-6) which efficiently cross the blood-brain barrier (BBB), Angiopep-7 is not efficiently transported across the BBB.

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Aprotinin-like polypeptides for delivering agents conjugated thereto to tissues

Background of the Invention

The invention relates to improvements in the field of drug delivery. More particularly, the invention relates to polypeptides, conjugates and pharmaceutical compositions including the polypeptides of the invention and their use for transporting agents (e.g., therapeutic agents) across into particular cell types such as liver, lung, or kidney.

Diseases of the liver, including hepatitis (e.g., viral hepatitis) and cancers of the liver (e.g., hepatocarcinoma), and lung diseases, such as lung cancer (e.g., small cell and non-small cell lung cancer) are serious health problems. Many therapeutic agents for such diseases have undesirable side effects (e.g., chemotherapeutic agents) or, for reasons such as in vivo stability, transport, or other pharmacokinetic properties, are difficult to provide at a sufficiently high concentration in the target tissue or for a sufficiently long duration to allow maximal therapeutic effect in the target tissue.

Accordingly, there is a need for methods and compositions that increase concentrations of therapeutic and diagnostic agents in target organs or tissues.

SUMMARY OF THE INVENTION

We have discovered that the polypeptides described herein (e.g., Angiopep-7; SEQ ID NO:112) are efficiently transported into particular cell types (e.g., liver, lung, spleen, kidney, and muscle), but are not efficiently transported across the blood-brain barrier (BBB). When conjugated to an agent, the polypeptides act as vectors and are capable of increasing the concentration of the conjugated agent in the cell. We have also identified Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, and Angiopep-6 (SEQ ID NOS:107-111) as vectors which can be efficiently transported across the BBB and may

additionally be transported into particular cell types. The invention therefore features polypeptides, conjugates including the polypeptides as vectors, and methods for diagnosing and treating diseases (e.g., cancer) with such polypeptides and conjugates.

Accordingly, in a first aspect the invention features a polypeptide including an amino acid sequence having the formula:

X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-X17-X18-X19

where each of X1-X19 (e.g., X1-X6, X8, X9, X11-X14, and X16-X19) is, independently, any amino acid (e.g., a naturally occurring amino acid such as Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) or absent and at least one of X1, X10, and X15 is arginine. In some embodiments, X7 is Ser or Cys; or X10 and X15 each are independently Arg or Lys. In some embodiments, the residues from X1 through X19, inclusive, are substantially identical to any of the amino acid sequences of any one of SEQ ID NOS:1-105 and 107-112 (e.g., Angiopep-1, Angiopep-2, Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7). In some embodiments at least one (e.g., 2, 3, 4, or 5) of the amino acids X1-X19 are Arg (e.g., any one, two, or three of X1, X10, and X15).

In other embodiments, the invention features a polypeptide including an amino acid sequence having substantial identity to any one of SEQ ID NOS:1-105 and 107-112 (e.g., Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6 and Angiopep-7). In certain embodiments, the polypeptide may include or may be Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, or Angiopep-7 (SEQ ID NOS:107-112), may include a sequence substantially identical to the Angiopep-7 sequence or a fragment thereof (e.g., a functional fragment). The polypeptide may have an amino acid sequence of 10 to 50 amino acids, e.g., 10 to 30 amino acids, in length. The invention also

features functional derivatives (e.g., chemical derivatives or variants) of these polypeptides.

Exemplary polypeptides of the invention have a lysine or arginine at position 10 (with respect to amino acid sequence of SEQ ID NO:1) or a lysine or arginine at position 15 (with respect to amino acid sequence of SEQ ID NO:1), or a lysine or arginine at both position 10 and at position 15. The polypeptides of the invention may also have a serine or cysteine at position 7 (with respect to amino acid sequence of SEQ ID NO:1). Where multimerization of polypeptides is desired, the polypeptide may include a cysteine (e.g., at position 7).

In certain embodiments, the polypeptides of the invention (e.g., any polypeptide described herein) are modified (e.g., as described herein). The polypeptide may be amidated, acetylated, or both. For example, a polypeptide including Angiopep-6 or Angiopep-7 may be amidated, or SEQ ID NO:67 may be amidated (polypeptide No. 67). In another example, the amino acid of any one of SEQ ID NOS:107-112, or any other polypeptide of the invention, is amidated or acetylated. Such modifications to polypeptides of the invention may be at the amino or carboxy terminus of said polypeptide. The invention also features peptidomimetics (e.g., those described herein) of any of the polypeptides described herein. The polypeptides of the invention may be in a multimeric form. For example, polypeptides may be in a dimeric form (e.g., formed by disulfide bonding through cysteine residues).

The polypeptides of the invention may be efficiently transported into particular cells (e.g., liver, kidney, lung, muscle, or spleen cells) or may efficiently cross the BBB (e.g., Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, and Angiopep-6). In some embodiments, the polypeptide are efficiently transported into particular cells (e.g., liver, kidney, lung, muscle, or spleen cells) and are not efficiently transported across the BBB (e.g., Angiopep-7). The polypeptide may be efficiently transported into at least one

(e.g., at least two, three, four, or five) of a cell or tissue selected from the group consisting of liver, kidney, lung, muscle, or spleen.

For any of the polypeptides and conjugates described herein, the amino acid sequence may specifically exclude a polypeptide including or consisting of any of SEQ ID NOS:1-105 and 107-112 (e.g., any of SEQ ID NOS:1-96, Angiopep-1, Angiopep-2, Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7). In some embodiments, the polypeptides and conjugates of the invention exclude the polypeptides of SEQ ID NOS:102, 103, 104 and 105. In other embodiments, the polypeptides and conjugates of the invention include these polypeptides.

In certain embodiments, a polypeptide of the invention may have an amino acid sequence described herein with at least one amino acid substitution (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 substitutions). In certain embodiments, the polypeptide may have an arginine at one, two, or three of the positions corresponding to positions 1, 10, and 15 of the amino acid sequence of any of SEQ ID NO:1, Angiopep-1, Angiopep-2, Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7. For example, the polypeptide may contain 1 to 12 amino acid substitutions (e.g., SEQ ID NO:91). For example, the amino acid sequence may contain 1 to 10 (e.g., 9, 8, 7, 6, 5, 4, 3, 2) amino acid substitutions or 1 to 5 amino acid substitutions. In accordance with the invention, the amino acid substitution may be a conservative or non-conservative amino acid substitution.

The polypeptides of the invention may be synthesized chemically (e.g., solid phase synthesis) or may be produced by recombinant DNA technology, as known in the art. Any of the polypeptides, compositions, or conjugates described herein may be in an isolated form or in a substantially purified form.

In another aspect, the invention features a polynucleotide sequence encoding a polypeptide of the invention (e.g., any polypeptide described herein, such as Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7). More particularly, nucleotide sequences (deoxyribonucleotides,

ribonucleotides, or derivatives thereof) encoding a polypeptide selected from the group consisting of any one of SEQ ID NOS:1-97 and SEQ ID NO:107-112 are encompassed by the invention. A desired nucleotide sequence may be synthesized chemically by methods known in the art.

In another aspect, the invention features a conjugate including a vector selected from the group consisting of any one of the polypeptides, analogs, or derivatives thereof described herein (e.g., Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7), and an agent, where the agent is conjugated to the vector.

The agent may be selected from the group consisting of a therapeutic agent (e.g., a small molecule drug, such as an anticancer agent, antibiotic, or any described herein), a detectable label, a polypeptide (e.g., an enzyme), and a protein complex. In some embodiments, the agent has a maximum molecular weight of about 160,000 Daltons. The agent may be a molecule active in the central nervous system. The agent may be any agent useful for treating or detecting a neurological, liver, lung, kidney, or spleen disease. The detectable label may be, for example, a radioimaging agent (e.g., an isotope), a fluorescent label (e.g., rhodamine, FITC, cy5.5, alexa), a reporter molecule (e.g., biotin). Other examples of detectable labels include green fluorescent protein, histag protein, and β-galactosidase. The conjugate may be a fusion protein consisting essentially of the vector and a protein. Examples of protein-based compounds which can be conjugated to a vector of the invention include an antibody (e.g., including heavy and/or light chains), an antibody fragment (e.g., an antibody binding fragment such as Fv fragment, F(ab)2, F(ab)2', and Fab). An antibody or fragment thereof that may be conjugated to the vector includes a monoclonal or polyclonal antibody and, further, may be of any origin (e.g., human, chimeric, and humanized antibodies). Other protein or protein-based compounds include cellular toxins (e.g., monomethyl auristatin E (MMAE), bacteria endotoxins and exotoxins, diphtheria toxins, botulinum toxin, tetanus toxins, perussis toxins, staphylococcus enterotoxins, toxin shock syndrome

toxin TSST-1, adenylate cyclase toxin, shiga toxin, and cholera enterotoxin) and anti-angiogenic compounds (endostatin, catechins, nutriceuticals, chemokine IP-10, inhibitors of matrix metalloproteinase (MMPIs), anastellin, vironectin, antithrombin, tyrosine kinase inhibitors, VEGF inhibitors, antibodies against receptor, herceptin, avastin, and panitumumab).

Leptin exendin-4, GLP-1, PYY, or PYY(3-36) may be used, e.g., for treatment of obesity. Other polypeptides that may be included in a conjugate of the invention are adrenocortiocotropic hormones (ACTH, corticotropin), growth hormone peptides (e.g., human placental lactogen (hPL), growth hormones, and prolactin (Prl)), melanocyte stimulating hormones (MSH), oxytocin, vasopressin (ADH), corticotropin releasing factor (CRF), gonadotropin releasing hormone associated peptides (GAP), growth hormone releasing factor (GRF), lutenizing hormone release hormones (LH-RH), orexins, prolactin releasing peptides, somatostatin, thyrotropin releasing hormone (THR), calcitonin (CT), caltitonin precursor peptide, calcitonins gene related peptide (CGRP), parathyroid hormones (PTH), parathyroid hormone related proteins (PTHrP), amylin, glucagon, insulin and insulin-like peptides, neuropeptide Y, pancreatic polypeptide (PP), peptide YY, somatostatin, cholecystokinin (CCK), gastrin releasing peptide (GRP), gastrin, gastrin inhibitory peptide, motilin, secretin, vasoactive intestinal peptide (VIP), natriuretic peptides (e.g., atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), brain natriuretic peptide, and C-type natriuretic peptide (CNP)), tachykinins (e.g., neurokinin A, neurokinin B, and substance P), substance P, angiotensins (e.g., angiotensin I and angiotensin II), renin, endothelins (e.g., endothelin-1, endothelin-2, endothelin-3, sarafotoxin (a snake venom) and scorpion toxin), sarafotoxin peptides, opioid peptides (e.g., casomorphin peptides, demorphins, endorphins, enkephalins, deltorphins, dynorphins), thymic peptides (e.g., thymopoietin, thymulin, thymopentin, thymosin, thymic humoral factor (THF)), adrenomedullin peptides (AM), allatostatin peptides, amyloid beta-protein fragments (Aβ fragments), antimicrobial peptides (e.g.,

defensin, cecropin, buforin, and magainin), antioxidant peptides (e.g., natural killer-enhancing factor B (NKEF-B), bombesin, bone Gla protein peptides (e.g., osteocalcin (bone Gla-protein, or BGP), CART peptides, cell adhesion peptides, cortistatin peptides, fibronectin fragments and fibrin related peptides, FMRF peptides, galanin, guanylin and uroguanylin, and inhibin peptides.

Small molecule drugs include an anticancer agents (e.g., any such agent described herein). Examples of anticancer agents include paclitaxel (Taxol), vinblastine, vincristine, etoposide, doxorubicin, cyclophosphamide, taxotere, melphalan, chlorambucil, and any anticancer agent described herein, or any combination thereof. An anticancer agent may have chemical moiety allowing conjugation to the vector of the invention.

In certain embodiments, the conjugate of the invention may include the formula V_x-L_y-A_z or a pharmaceutically acceptable salt thereof, wherein V is a polypeptide or derivative thereof described herein (e.g., Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, Angiopep-7, or any of the analogs, derivatives, or fragments described herein). V may be efficiently transported into a particular cell type (e.g., a liver, lung, kidney, spleen, or muscle cell) or may be efficiently transported across the BBB (e.g., after attachment to L_y-A_z). In certain embodiments, the vector or conjugate is not efficiently transported across the BBB, but is efficiently transported into a particular cell type (e.g., Angiopep-7). L is a linker or a bond (e.g., chemical or covalent bond). A is an agent such as those selected from the group consisting of a therapeutic agent (e.g., a small molecule drug), a detectable label, a protein, or protein-based compound (e.g., antibody, an antibody fragment), an antibiotic, an anti-cancer agent, an anti-angiogenic compound and a polypeptide or any molecule active at the level of the central nervous system.

X, Y, and Z may each independently be any number greater than 0 (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) or may represent a range of values between 1 (e.g., 2, 3, 4, 5, 6, 7, 8, 9) and 10 (e.g., 9, 8, 7, 6, 5, 4, 3, 2). Accordingly, the formula V_x-L_y-A_z is not restricted to a specific order or specific ratio; for

example, the conjugate may include 1 to 5 vectors (1, 2, 3, 4, or 5) coupled to the agent. In other embodiments, more than one agent may be conjugated to the vector. The agent or conjugate may be active when the agent is conjugated to the vector. In some embodiments, the compound can be released from the vector, e.g., following transport across the BBB or into a particular cell type. The compound may become active following its release (e.g., as a prodrug). In some embodiments, the agent remains conjugated to the vector following transport. Here, the conjugate may exhibit reduced efflux transport (e.g., by P-glycoprotein) as compared to the unconjugated agent. In this case, it may be desirable that the agent and vector remain bound. The conjugate may be provided in a pharmaceutically acceptable composition.

The agent, when conjugated to a vector, may exhibit modified (e.g., improved) bioavailability, improved potency (e.g., anticancer activity), altered tissue distribution of the agent, decreased toxicity, or altered pharmacokinetics as compared to the agent when unconjugated to the vector. A vector (e.g., Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, and Angiopep-6) may promote accumulation of the agent in the brain of an individual (e.g., a brain containing a tumor cell such as a glioblastoma, lung, breast, colon, liver, pancreas, or spleen, tumor cell) or in a particular cell or tissue (e.g., liver, lung, kidney, spleen, and muscle). A vector such as Angiopep-7 may be able to promote accumulation in cell types such as liver, lung, kidney, spleen or muscle, but not promote accumulation in the brain. More particularly, the vector can increase the accumulation or potency of an agent which is a P-gp substrate or therapeutic agents which are expelled by P-gp or a P-gp-related protein (e.g., a P-gp human or mammalian allelic variant, e.g., a mdr1a or mdr1b isoforms from a rodent), when provided (e.g., administered) to a subject. The cell may express or may be able to express P-gp or P-gp related protein. The cell into which the conjugate is transported may express a vector receptor or transporter, for example, a low-density lipoprotein related receptor (LRP) (e.g., a cell which co-expresses P-gp or a P-gp related protein). The cell may

be, for example a normal cell, a tumor cell, or a metastatic cell (e.g., a multidrug resistance cancer cell). The cell or tumor may be a metastasis (e.g., of any of the cancers described herein).

A vector or conjugate may be efficiently transported into a particular cell type (e.g., liver, lung, kidney, spleen, or muscle cells) and may be efficiently transported across the BBB; such conjugates include Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, and Angiopep-6. In certain embodiments, the transporting activity of the vector, per se, does not affect the integrity of the cell into which it is being delivered or does not affect BBB integrity. In other embodiments, the vector or conjugate, such as Angiopep-7, is efficiently transported into a particular cell type, but is not efficiently transported across the BBB. Any vector or conjugate described here may be transported by receptor-mediated endocytosis or transcytosis or by adsorptive-mediated endocytosis or transcytosis.

Any vector or conjugate described herein may be present in an pharmaceutically acceptable carrier (e.g., may be in the form a medicament). The invention therefore features a pharmaceutical composition including (a) a conjugate (e.g., any conjugate described herein); (b) a pharmaceutically acceptable carrier (e.g., any described herein). The conjugate may further include (c) a solubilizer. The solubilizer may be, for example, a polyoxyethylene ester of fatty acid (e.g., Solutol® HS-15). The pharmaceutical composition may be used for modifying the pharmacokinetics of a compound, for reducing the growth of a tumor cell, or for the detection of a tumor cell.

In another aspect, the invention features the use of a vector or a conjugate of the invention for the diagnosis or treatment of (e.g., for the manufacture of a medicament or use in treating) a liver disease, lung disease, kidney disease, spleen disease, or muscle disease. In certain embodiments a neurological disease or a central nervous system disease (e.g., any disease described herein) may be treated using a conjugate of the invention that is efficiently transported across the BBB. For example, the vector or conjugate

may be used for the *in vivo* detection of any of these diseases. The treatment or diagnosis may be performed in a mammal (e.g., a human or non-human mammal) in need thereof, such as a mammal having or at risk (e.g., increased risk) of having the disease (e.g., a cancer). The administration may be performed using any means known in the art, for example, intraarterially, intranasally, intraperitoneally, intravenously, intramuscularly, subcutaneously, transdermally, or *per os*. The administration may be in a therapeutically effective amount.

By conjugating an agent to a vector described herein, the toxicity of the agent may be reduced, thus allowing the agent to be provided to the subject at a dose higher than the recommended dose for the agent alone. Accordingly, the invention features a method of treating a subject having a disease or condition (e.g., any disease or condition described herein such as cancer) including providing a conjugate of the invention to the subject, wherein said conjugate includes an agent, at a dose higher (e.g., at least 5%, 10%, 25%, 50%, 75%, 100%, 250%, 500%, 1,000%, 5,000%, or 10,000% higher) than the therapeutic dose of the agent alone.

Because a vector described herein may be capable of targeting an agent to a particular cell type (e.g., those described herein), the agent when conjugated to a vector can, in certain embodiments, have a higher efficacy when administered to the subject. Accordingly, the invention also features a method of treating a subject having a disease or condition (e.g., any disease or condition described herein such as cancer) by administering to the subject a conjugate or a composition including a conjugate of the invention, wherein the conjugate includes agent, in a dose lower (e.g., 5%, 10%, 15%, 20%, 30%, 50%, 70%, 80%, 90%, 95%, 98%, 99%, 99.9% lower) than the therapeutic dose of the agent alone.

The treatments methods of the invention may further comprise a step of assessing whether the tumor of the individual includes a multiple drug resistant tumor cell (e.g., a cell expressing P-gp (MDR1) or determining whether the

tumor may have or has a multiple resistance drug phenotype). The treatment methods may include a step of providing chemotherapy, radiotherapy, or both. Alternatively, the subject being treated may have received (or will receive) such therapies (e.g., within 1 year, 6 mo, 3 mo, 2 mo, 1 mo, 2 weeks, 1 week, 3 days, 2 days, or 1 day). The individual may have had surgery. The individual may have a brain tumor (e.g., a primary brain tumor) or a tumor at a site other than the brain (e.g., does not have a brain tumor). An individual in need may have present or is at risk of developing a resistance to at least one drug (e.g., a multiple drug resistance (MDR) phenotype). The tumor may be a lung tumor, an extracranial metastasis of a brain tumor of (e.g., from a glioblastoma), a brain tumor of metastatic origin (e.g., from a lung tumor, a breast tumor, a melanoma, a colorectal cancer, or a urinary organ tumor). The tumor may include a cell expressing P-gp, LRP, or both, or a cell capable of expressing P-gp, LRP, or both. P-gp and LRP may be localized to a cell surface.

In other aspect, the invention also features a method of increasing the concentration of an agent in a cell or transport of an agent to a cell, where the cell expresses a member of the LRP receptor family. The method includes contacting the cell with a conjugate comprising the agent conjugated to a vector (e.g., any polypeptide described herein), thereby increasing concentration of the agent in the cell, or transporting the agent to the cell. The invention also features a method of increasing the concentration of agent in a cell, where the cell expresses P-glycoprotein. The method includes contact the cell with a conjugate including the agent conjugated to a vector (e.g., any polypeptide described herein), thereby increasing the concentration of the agent in the cell, as compared to the unconjugated agent.

In accordance with the any of the aspects of the invention, neurological diseases include a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke, and BBB related malfunctions (e.g., obesity). The liver disease may be a cancer

such as hepatocarcinoma. Other liver diseases include those described herein. The lung disease may be a lung cancer (e.g., those described herein).

The conjugates can include vectors in the form of multimers such as dimers. Multimeric forms of the vectors can, in some embodiments, increase the transport or accumulation of the agent. Vectors may also be in a ratio of at least 1:1 (agent:vector), 1:2, 1:3, 1:4, 1:5, 1:6, or 1:10. As indicated herein, the higher ratios of vector to agent can lead to increased transport. As such, the number of vector per agent is not intended to be limited.

Also in accordance with the invention, the pharmaceutical composition may be used, for example, for the delivery of an agent to the CNS of an individual.

A pharmaceutically acceptable salt of a vector (polypeptide) or of a conjugate is encompassed by the invention and may include pharmaceutically acceptable acid addition salts of the agent.

The vector or conjugate of the invention may be used in combination with or separately from conventional methods of treatment or therapy. Combination therapy with other agents may include sequential or concurrent administration to an individual. Pharmaceutical compositions of the invention may include a combination of a vector-agent conjugate of the invention in association with a pharmaceutically acceptable excipient, as described herein, and another therapeutic or prophylactic agent known in the art.

By "vector" is meant a compound or molecule such as a polypeptide that is able to be transported into a particular cell type (e.g., liver, lungs, kidney, spleen, or muscle) or across the BBB. The vector may be attached to (covalently or not) or conjugated to an agent and thereby may be able to transport the agent into a particular cell type or across the BBB. In certain embodiments, the vector may bind to receptors present on cancer cells or brain endothelial cells and thereby be transported into the cancer cell or across the BBB by transcytosis. The vector may be a molecule for which high levels of transendothelial transport may be obtained, without affecting the cell or BBB

integrity. The vector may be a polypeptide or a peptidomimetic and may be naturally occurring or produced by chemical synthesis or recombinant genetic technology.

By "conjugate" is meant a vector linked to an agent. The conjugation may be chemical in nature, such as via a linker, or genetic in nature for example by recombinant genetic technology, such as in a fusion protein with for example a reporter molecule (e.g., green fluorescent protein, β-galactosidase, Histag, etc.).

By a vector which is "efficiently transported across the BBB" is meant a vector that is able to cross the BBB at least as efficiently as Angiopep-6 (i.e., greater than 38.5% that of Angiopep-1 (250 nM) in the in situ brain perfusion assay described herein). Accordingly, a vector or conjugate which is "not efficiently transported across the BBB" is transported to the brain at lower levels (e.g., transported less efficiently than Angiopep-6).

By a vector or conjugate which is "efficiently transported to a particular cell type" is meant a vector or conjugate that is able to accumulate (e.g., either due to increased transport into the cell, decreased efflux from the cell, or a combination thereof) in that cell type at least 10% (e.g., 25%, 50%, 100%, 200%, 500%, 1,000%, 5,000%, or 10,000%) greater extent than either a control substance, or, in the case of a conjugate, as compared to the unconjugated agent.

By "substantially pure" or "isolated" is meant a compound (e.g., a polypeptide or conjugate) that has been separated from other chemical components. Typically, the compound is substantially pure when it is at least 30%, by weight, free from other components. In certain embodiments, the preparation is at least 50%, 60%, 75%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% by weight, free from other components. A purified polypeptide may be obtained, for example, by expression of a recombinant polynucleotide encoding such a polypeptide or by chemically synthesizing the polypeptide. Purity can be measured by any appropriate method, for example, column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

By "analogue" is meant a polypeptide originating from an original sequence or from a portion of an original sequence and which may include one or more modification; for example, one or more modification in the amino acid sequence (e.g., an amino acid addition, deletion, insertion, or substitution), one or more modification in the backbone or side-chain of one or more amino acid, or an addition of a group or another molecule to one or more amino acids (side-chains or backbone). An analog may have one or more amino acid insertions, at either or at both of the ends of the polypeptide or inside the amino acid sequence of the polypeptide. An analogue may have sequence similarity and/or sequence identity (e.g., may be substantially identical) with that of an original sequence or a portion of an original sequence. Analogs may include a modification of its structure, e.g., as described herein. The degree of similarity between two sequences is base upon the percentage of identities (identical amino acids) and of conservative substitution. An analogue may have at least 35%, 50f%, 60%, 70%, 80%, 90%, or 95% (e.g., 96%, 97%, 98%, 99%, and 100%) sequence similarity to an original sequence with a combination of one or more modifications in a backbone or side-chain of an amino acid, or an addition of a group or another molecule. Exemplary amino acids which are intended to be similar (a conservative amino acid) to others are known in the art and includes, for example, those listed in Table 3.

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 35%, 40%, 50%, 55%, 60%, 65%, 70%, 75%, 85%, 90%, 95%, or even 99% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 4 (e.g., at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 50, or 100) amino acids. For nucleic acids, the length of comparison sequences will generally be at least 60 nucleotides, preferably at least 90 nucleotides, and more preferably at least 120 nucleotides, or full length. It is to be understood herein that gaps may be found between the amino acids of an analogs which are identical or similar to amino acids of the original polypeptide. The gaps may

include no amino acids, one or more amino acids which are not identical or similar to the original polypeptide. Biologically active analogs of the vectors (polypeptides) of the invention are encompassed herewith. Percent identity may be determined, for example, with n algorithm GAP, BESTFIT, or FASTA in the Wisconsin Genetics Software Package Release 7.0, using default gap weights.

By "functional derivative" is meant a "chemical derivative," "fragment," or "variant" biologically active sequence or portion of a vector or agent or conjugate and a salt thereof of the invention. A vector functional derivative may be able to be attached to or conjugated to an agent and enter a particular cell type, thereby transporting the agent into that cell.

By "chemical derivative" is meant a vector, an agent, or a conjugate of the invention, which contains additional chemical moieties not a part of the vector, agent or vector-agent conjugate, including covalent modifications. A chemical derivative may be prepared by direct chemical synthesis using methods known in the art. Such modifications may be introduced into a protein or peptide vector, agent, or vector-agent conjugate by reacting targeted amino acid residues with an organic derivatizing agent capable of reacting with selected side chains or terminal residues. A vector chemical derivative may be able to cross the BBB or to enter or accumulate in a particular cell type (e.g., those described herein). In a preferred embodiment, very high levels of transendothelial transport across the BBB are obtained without effecting BBB integrity.

By "fragment" is meant a polypeptide originating from a portion of an original or parent sequence or from an analogue of said parent sequence. Fragments encompass polypeptides having truncations of one or more amino acids, wherein the truncation may originate from the amino terminus (N-terminus), carboxy terminus (C-terminus), or from the interior of the protein. A fragment may include the same sequence as the corresponding portion of the original sequence. Functional fragments of the vector (polypeptide) described

herein are encompassed by the invention. Fragments may be at least 5 (e.g., at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 28, 30, 35, 40, 45, 50, 60, 75, 100, or 150) amino acids. Fragments of the invention may include, for example, a polypeptide of 7, 8, 9 or 10 amino acids to 18 amino acids. Fragments may contain any of the modifications described herein (e.g., acetylation, amidation, amino acid substitutions)

A "non-naturally occurring amino acid" is an amino acid which is not naturally produced or found in a mammal.

By "agent" is meant, any compound, for example, an antibody, or a therapeutic agent, a marker, a tracer, or an imaging compound.

By "therapeutic agent" is meant an agent having a biological activity. In some cases, the therapeutic agent is used to treat the symptoms of a disease, a physical or mental condition, an injury, or an infection and includes anti-cancer agents, antibiotics, anti-angiogenic agents, and molecules active at the level of the central nervous system.

By "small molecule drug" is meant a drug having a molecular weight of 1,000 g/mol or less (e.g., less than 800, 600, 500, 400, or 200 g/mol).

By "subject" is meant a human or non-human animal (e.g., a mammal).

By "treatment," "treating," and the like are meant obtaining a desired pharmacological or physiological effect, e.g., inhibition of cancer cell growth, death of a cancer cell, amelioration of a disease or condition (e.g., any disease or condition described herein), or amelioration of at least one symptom associated with a disease or condition. The effect may be prophylactic, e.g., completely or partially preventing a disease or symptom thereof or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. Treatment includes: (a) preventing a disease or condition (e.g., preventing cancer) from occurring in an individual (e.g., one who is predisposed to the disease but has not been diagnosed as having it); (b) inhibiting a disease (e.g., arresting its development); or (c) relieving a disease (e.g., reducing symptoms associated with a disease). Treatment includes any

administration of a therapeutic agent to an individual to treat, cure, alleviate, improve, diminish or inhibit a condition in the individual, including, without limitation, administering a vector-agent conjugate to an individual.

By "cancer" is meant any cellular proliferation whose unique trait is the loss of normal controls which can result in unregulated growth, lack of differentiation, or ability to invade tissues and metastasize. Cancer can develop in any tissue or in any organ. Cancer is intended to include, without limitation, cancer of the brain, liver, lungs, kidney, or spleen. Additional cancers are described herein.

By "providing" is meant, in the context of a vector or conjugate of the invention, to bring the vector or conjugate into contact with a target cell or tissue either in vivo or in vitro. A vector or conjugate may be provided by administering the vector or conjugate to a subject.

By "administering" and "administration" is meant a mode of delivery including, without limitation, intra-arterially, intra-nasally, intraperitoneally, intravenously, intramuscularly, subcutaneously, transdermally, or *per os*. A daily dosage can be divided into one, two, or more doses in a suitable form to be administered at one, two or more times throughout a time period.

By "therapeutically effective" or "effective amount" is meant an amount of a therapeutic agent sufficient to improve, decrease, prevent, delay, suppress, or arrest any symptom of the disease or condition being treated. A therapeutically effective amount of an agent need not cure a disease or condition but will provide a treatment for a disease or condition such that the onset of the disease or condition is delayed, hindered, or prevented, or the disease or condition symptoms are ameliorated, or the term of the disease or condition is changed or, for example, is less severe or recovery is accelerated in an individual.

By "condition" is meant any situation causing pain, discomfort, sickness, disease or disability (mental or physical) to or in an individual, including neurological disease, injury, infection, or chronic or acute pain. Neurological

diseases include brain tumors, brain metastases, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, and stroke.

By "pharmaceutical composition" is meant a therapeutically effective amount of an agent together with a pharmaceutically acceptable diluent, preservative, solubilizer, emulsifier, or adjuvant, for example, any of those described herein.

By "therapeutic dose" is meant the dosage of a agent such as a drug (without the vector) acceptable for use clinically with respect to its toxicity or efficacy. By conjugation of an agent to a vector of the invention, it may be possible to administer the agent at a dosage either lower or higher dosage than the therapeutic dose.

If a "range" or "group of substances" is mentioned with respect to a particular characteristic (e.g., temperature, concentration, time and the like), the invention relates to and explicitly incorporates herein each and every specific member and combination of sub-ranges or sub-groups therein. Thus, for example, with respect to a length of from 9 to 18 amino acids, is to be understood as specifically incorporating herein each and every individual length, e.g., a length of 18, 17, 15, 10, 9, and any number therebetween. Therefore, unless specifically mentioned, every range mentioned herein is to be understood as being inclusive. For example, in the expression from 5 to 19 amino acids long is to be as including 5 and 19. This similarly applies with respect to other parameters such as sequences, length, concentrations, elements, and the like.

The sequences, regions, portions defined herein each include each and every individual sequence, region, and portion described thereby as well as each and every possible sub-sequence, sub-region, and sub-portion whether such sub-sequences, sub-regions, and sub-portions are defined as positively including particular possibilities, as excluding particular possibilities or a combination thereof. For example, an exclusionary definition for a region may read as follows: "provided that said polypeptide is no shorter than 4, 5, 6, 7, 8

or 9 amino acids. A further example of a negative limitation is the following; a sequence including SEQ ID NO:X with the exclusion of a polypeptide of SEQ ID NO:Y; etc. An additional example of a negative limitation is the following; provided that said polypeptide is not (does not include or consist of) SEQ ID NO:Z.

Brief Description of the Drawings

Fig. 1 illustrates the structure of exemplary polypeptides of the invention.

Fig. 2 illustrates the protocol used to conjugate aprotinin with IgG using cross-linker BS^3.

Fig. 3 illustrates the protocol used to conjugate aprotinin with IgG using cross-linker sulfo-EMCS.

Fig. 4 illustrates the protocol used to conjugate Angiopep-2 with antibodies using cross-linker ECMS.

Fig. 5 illustrates the protocol used to conjugate Angiopep-2 with antibodies using cross-linker SATA.

Fig. 6 illustrates the protocol used to conjugate Angiopep-2 with antibodies using carbohydrate targets via hydrazide.

Fig. 7 illustrates other possible linkers which may be used in the making of a conjugate.

Fig. 8 illustrates a method for attaching a vector of the invention to paclitaxel.

Fig. 9 is a schematic representation of the efflux pump, P-glycoprotein (P-gp or MDR1) at the cell surface. The efflux pump, P-gp or MDR1, associated with multidrug resistance is highly expressed at the cell surface of many cancer cells and various tissues including the blood-brain barrier (BBB).

Fig. 10A and 10B are diagrams representing tissue distribution of Taxol and TxlAnl conjugate.

Fig. 11 is a diagram representing lung distribution of Taxol and TxlAn1.

Fig. 12 is a diagram representing the levels of TxlAn1 conjugate in plasma and lung.

Fig. 13 is a set of images showing in vivo accumulation of Angiopep-2 and Angiopep-7 in the brains of rats 30 minutes following IV injection. Angiopep-2 accumulates to a greater extent in the brain as compared to Angiopep-7.

Fig. 14 is a set of images showing fluorescence microscopy of brain sections following a 10 min in situ perfusion of either fluorescently labeled Angiopep-2 or Angiopep-7. These images show that Angiopep-2 is localized within the brain, whereas Angiopep-7 is localized within the capillaries.

Fig. 15A is a set of images showing in vivo imaging of Angiopep-2 and Angiopep-7 in the liver, lungs, and kidneys of a rat. Angiopep-7 is observed to accumulate in these tissues.

Fig. 15B is a graph showing ex-vivo organ imaging 24 hours following injection of either Angiopep-2 or Angiopep-7 in a rat. Both polypeptides accumulate in the kidneys, liver, and lungs. Angiopep-2 accumulates to a greater degree in the brain as compared to Angiopep-7.

Fig. 16 illustrates the distribution volume in the brain parenchyma of free and Angiopeps conjugated IgG.

Fig. 17 is a diagram of cell proliferation in the presence of the parent drug Taxol. Glioblastoma cells (U-87) were exposed to various concentrations of Taxol for 3-days. ^{3}H- Thymidine incorporated in cells were plotted as a function of Taxol concentrations.

Figs. 18A and 18B are graphs representing the effect of TxlAn2 treatment on subcutaneous glioblastomas (U-87) tumor growth.

Fig. 19 is a set of photomicrographs showing detection of β-tubulin in NCI-H460 cells by immunofluoresence or visible light in cancer cells exposed to Taxol or TxlAn2 conjugate. As a control, cells were exposed to 1% DMSO.

Fig. 20 is a set of graphs showing the effect of Taxol and TxlAn2 conjugate on NCI-H460 cell-cycle measured by FACS. Cells were exposed for

24 hrs with the vehicle (DMSO), Taxol (100 nM), or TxlAn2 conjugate (30 nM, equivalent to 100 nM of Taxol).

Figs. 21A is a graph showing accumulation of various drugs in MDCK cells transfected with MDRl in the presence or absence (control) of 10 μM CsA, a P-gp inhibitor. The experiment was performed in the presence of 1% DMSO.

Fig. 21B is a graph showing accumulation of the conjugate in cells over-expressing P-gp.

Fig. 22A and 22B are photographs of western blots showing immunodetection of LRP in human brain tumor biopsies.

Figs. 23A and 23B are a schematic representation of conjugation of a drug to the vector of the invention.

Fig. 24 is a chromatogram illustrating production of the TxlAn2 (3:1) conjugate.

Fig. 25 is a HPLC analysis of the peak purified on a hydrophobic column using AKTA- explorer.

Fig. 26 illustrates the association of Angiopep-2 with the light and heavy chain of IgG.

Fig. 27 illustrates increased brain distribution volume of IgG-Angiopep-2 conjugates cross-linked with sulfo-EMCS.

Fig. 28 illustrates the brain penetration for IgG-Angiopep-2 conjugates using *in situ* brain perfusion.

Fig. 29 illustrates an autoradiogram of radiolabeled [^{125}I]-IgG-Angiopep conjugates.

Fig. 30 illustrates the similar detection of EGFR on U87 cells with anti-EGFR and anti-EGFR-Angiopep-2 conjugate by FACS analysis.

Fig. 31 illustrates the distribution volume in the brain parenchyma of free and Angiopep-2 conjugated EGFR antibody.

Fig. 32 illustrates the distribution volume in the brain parenchyma of free and Angiopep-2 conjugated VEFG antibody.

Fig. 33 illustrates the uptake of conjugates including different ratios of vector to the agent (antibody) in the parenchyma.

Detailed Description of the Invention

We have discovered that Angiopep-7 can be efficiently transported into particular cell types or organs such as liver, lungs, kidneys, spleen, and muscle, but is not efficiently transported across the blood-brain barrier (BBB), relative to polypeptides such as Angiopep-1 or Angiopep-2. On this basis we have identified Angiopep-7 as a vector suitable for transporting agents (e.g., for treatment of diseases associated with these tissues, such as cancer or any of the diseases described herein). We also have identified other polypeptides, including Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, and Angiopep-6, which also may be used as vectors in the invention. These polypeptides may retain the ability to be efficiently transported across the BBB, or to be transported into particular tissues or cell types (e.g., liver, lungs, kidneys, spleen, and muscle). For these polypeptides, agents which are unable or ineffective at efficiently crossing the BBB, may be transported across the BBB when conjugated to the polypeptide. Conjugates can be in the form of a composition, such as a pharmaceutical composition, for treatment or diagnosis of a condition or disease.

The conjugates of the invention (e.g., any of those described herein) may also have advantageous in vivo pharmacokinetic properties as compared to the agent alone. These properties may include increased half-life in vivo or increased concentration in, increased rate of accumulation in, or decreased removal from a desired cell type. On this basis, it may be possible to administer conjugates of the invention to a subject either at a lower dosage, less frequently, or for a shorter duration than the unconjugated agent. Lower amounts, frequency, or duration of administration may be especially desirable for therapeutics with known side effects. In other cases, the conjugating the agent to a polypeptide described herein may result in higher efficacy, due to

increased concentration in the target cells or tissue. Indeed, by concentrating the agent in a target cell type, unwanted side effects can be reduced (e.g., with increased efficacy). This may decrease the required duration of therapy. In some cases, it may even be possible to administer the conjugate at an increased dosage, frequency, or duration, relative to the dosage of the unconjugated agent and observe increased efficacy, reduced side effects, or both.

Polypeptides of the invention

The invention features any of polypeptides described herein, for example, any of the polypeptides described in Table 1 (e.g., a polypeptide defined in any of SEQ ID NOS:1-105 and 107-112 such as SEQ ID NOS:1-97, 99, 100, 101, or 107-112), or any fragment, analog, derivative, or variant thereof. In certain embodiments, the polypeptide may have at least 35%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or even 100% identity to a polypeptide described herein. The polypeptide may have one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15) substitutions relative to one of the sequences described herein. Other modifications are described in greater detail below.

The invention also features fragments of these polypeptides (e.g., a functional fragment). In certain embodiments, the fragments are capable of entering or accumulating in a particular cell type (e.g., liver, lung, kidney, spleen, or muscle) or capable of crossing the BBB. Truncations of the polypeptide may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more amino acids from either the N-terminus of the polypeptide, the C-terminus of the polypeptide, or a combination thereof. Other fragments include sequences where internal portions of the polypeptide are deleted.

Additional polypeptides may be identified by using one of the assays or methods described in U.S. Patent Application Publication No. 2006/0189515, which is hereby incorporated by reference, or by any method known in the art. For example, a candidate vector may be produced by conventional polypeptide synthesis, conjugated with Taxol and administered to a laboratory animal. A

biologically active vector may be identified, for example, based on its efficacy to increase survival of an animal injected with tumor cells and treated with the conjugate as compared to a control which has not been treated with a conjugate (e.g., treated with the unconjugated agent).

In another example, a biologically active polypeptide may be identified based on its location in the parenchyma in an in situ cerebral perfusion assay. In vitro BBB assays, such as the model developed by CELLIAL™ Technologies, may be used to identify such vectors.

Assays to determine accumulation in other tissues may be performed as well. See, for example, Example 1 herein. Labeled conjugates of a polypeptide can be administered to an animal, and accumulation in different organs can be measured. For example, a polypeptide conjugated to a detectable label (e.g., a near-IR fluorescence spectroscopy label such as Cy5.5) allows live in vivo visualization. Such a polypeptide can be administered to an animal, and the presence of the polypeptide in an organ can be detected, thus allowing determination of the rate and amount of accumulation of the polypeptide in the desired organ. In other embodiments, the polypeptide can be labeled with a radioactive isotope (e.g., ^{125}I). The polypeptide is then administered to an animal. After a period of time, the animal is sacrificed, and the animal's organs are extracted. The amount of radioisotope in each organ can then measured using any means known in the art. By comparing the amount of a labeled candidate polypeptide in a particular organ without amount of labeled control, the ability of the candidate polypeptide the rate or amount of accumulation of a candidate polypeptide in a particular tissue can be ascertained. Appropriate negative controls include any polypeptide known not be transported into a particular cell type.

TABLE 1

SEQ ID NO:																			
1	T	F	V	Y	G	G	C	R	A	K	R	N	N	F	K	S	A	E	D
2	T	F	Q	Y	G	G	C	M	G	N	G	N	N	F	V	T	E	K	E
3	P	F	F	Y	G	G	C	G	G	N	R	N	N	F	D	T	E	E	Y
4	S	F	Y	Y	G	G	C	L	G	N	K	N	N	Y	L	R	E	E	E
5	T	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	K	Y
6	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	R	A	K	Y
7	T	F	F	Y	G	G	C	R	A	K	K	N	N	Y	K	R	A	K	Y
8	T	F	F	Y	G	G	C	R	G	K	K	N	N	F	K	R	A	K	Y
9	T	F	Q	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	K	Y
10	T	F	Q	Y	G	G	C	R	G	K	K	N	N	F	K	R	A	K	Y
11	T	F	F	Y	G	G	C	L	G	K	R	N	N	F	K	R	A	K	Y
12	T	F	F	Y	G	G	S	L	G	K	R	N	N	F	K	R	A	K	Y
13	P	F	F	Y	G	G	C	G	G	K	K	N	N	F	K	R	A	K	Y
14	T	F	F	Y	G	G	C	R	G	K	G	N	N	Y	K	R	A	K	Y
15	P	F	F	Y	G	G	C	R	G	K	R	N	N	F	L	R	A	K	Y
16	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	R	E	K	Y
17	P	F	F	Y	G	G	C	R	A	K	K	N	N	F	K	R	A	K	E
18	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	R	A	K	D
19	T	F	F	Y	G	G	C	R	A	K	R	N	N	F	D	R	A	K	Y
20	T	F	F	Y	G	G	C	R	G	K	K	N	N	F	K	R	A	E	Y
21	P	F	F	Y	G	G	C	G	A	N	R	N	N	F	K	R	A	K	Y
22	T	F	F	Y	G	G	C	G	G	K	K	N	N	F	K	T	A	K	Y
23	T	F	F	Y	G	G	C	R	G	N	R	N	N	F	L	R	A	K	Y
24	T	F	F	Y	G	G	C	R	G	N	R	N	N	F	K	T	A	K	Y
25	T	F	F	Y	G	G	S	R	G	N	R	N	N	F	K	T	A	K	Y
26	T	F	F	Y	G	G	C	L	G	N	G	N	N	F	K	R	A	K	Y
27	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	L	R	A	K	Y
28	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	K	T	A	K	Y
29	T	F	F	Y	G	G	C	R	G	N	G	N	N	F	K	S	A	K	Y
30	T	F	F	Y	G	G	C	R	G	K	K	N	N	F	D	R	E	K	Y
31	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	L	R	E	K	E
32	T	F	F	Y	G	G	C	R	G	K	G	N	N	F	D	R	A	K	Y
33	T	F	F	Y	G	G	S	R	G	K	G	N	N	F	D	R	A	K	Y

TABLE 1

34	T	F	F	Y	G	G	C	R	G	N	G	N	N	F	V	T	A	K	Y		
35	P	F	F	Y	G	G	C	G	G	K	G	N	N	Y	V	T	A	K	Y		
36	T	F	F	Y	G	G	C	L	G	K	G	N	N	F	L	T	A	K	Y		
37	S	F	F	Y	G	G	C	L	G	N	K	N	N	F	L	T	A	K	Y		
38	T	F	F	Y	G	G	C	G	G	N	K	N	N	F	V	R	E	K	Y		
39	T	F	F	Y	G	G	C	M	G	N	K	N	N	F	V	R	E	K	Y		
40	T	F	F	Y	G	G	S	M	G	N	K	N	N	F	V	R	E	K	Y		
41	P	F	F	Y	G	G	C	L	G	N	R	N	N	Y	V	R	E	K	Y		
42	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	V	R	E	K	Y		
43	T	F	F	Y	G	G	C	L	G	N	K	N	N	Y	V	R	E	K	Y		
44	T	F	F	Y	G	G	C	G	G	N	G	N	N	F	L	T	A	K	Y		
45	T	F	F	Y	G	G	C	R	G	N	R	N	N	F	L	T	A	E	Y		
46	T	F	F	Y	G	G	C	R	G	N	G	N	N	F	K	S	A	E	Y		
47	P	F	F	Y	G	G	C	L	G	N	K	N	N	F	K	T	A	E	Y		
48	T	F	F	Y	G	G	C	R	G	N	R	N	N	F	K	T	E	E	Y		
49	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	D		
50	P	F	F	Y	G	G	C	G	G	N	G	N	N	F	V	R	E	K	Y		
51	S	F	F	Y	G	G	C	M	G	N	G	N	N	F	V	R	E	K	Y		
52	P	F	F	Y	G	G	C	G	G	N	G	N	N	F	L	R	E	K	Y		
53	T	F	F	Y	G	G	C	L	G	N	G	N	N	F	V	R	E	K	Y		
54	S	F	F	Y	G	G	C	L	G	N	G	N	N	Y	L	R	E	K	Y		
55	T	F	F	Y	G	G	S	L	G	N	G	N	N	F	V	R	E	K	Y		
56	T	F	F	Y	G	G	C	R	G	N	G	N	N	F	V	T	A	E	Y		
57	T	F	F	Y	G	G	C	L	G	K	G	N	N	F	V	S	A	E	Y		
58	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	D	R	A	E	Y		
59	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	L	R	E	E	Y		
60	T	F	F	Y	G	G	C	L	G	N	K	N	N	Y	L	R	E	E	Y		
61	P	F	F	Y	G	G	C	G	G	N	R	N	N	Y	L	R	E	E	Y		
62	P	F	F	Y	G	G	S	G	G	N	R	N	N	Y	L	R	E	E	Y		
63	M	R	P	D	F	C	L	E	P	P	Y	T	G	P	C	V	A	R	I		
64	A	R	I	I	R	Y	F	Y	N	A	K	A	G	L	C	Q	T	F	V	Y	G
65	Y	G	G	C	R	A	K	R	N	N	Y	K	S	A	E	D	C	M	R	T	C	G
66	P	D	F	C	L	E	P	P	Y	T	G	P	C	V	A	R	I	I	R	Y	F	Y
67	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y		
68	K	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y		

TABLE 1

69	T	F	Y	Y	G	G	C	R	G	K	R	N	N	Y	K	T	E	E	Y		
70	T	F	F	Y	G	G	S	R	G	K	R	N	N	F	K	T	E	E	Y		
71	C	T	F	F	Y	G	C	C	R	G	K	R	N	N	F	K	T	E	E	Y	
72	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y	C	
73	C	T	F	F	Y	G	S	C	R	G	K	R	N	N	F	K	T	E	E	Y	
74	T	F	F	Y	G	G	S	R	G	K	R	N	N	F	K	T	E	E	Y	C	
75	P	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y		
76	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	K	E	Y		
77	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	T	E	E	Y		
78	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	K	R	Y		
79	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	T	A	E	Y		
80	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	T	A	G	Y		
81	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	R	E	K	Y		
82	T	F	F	Y	G	G	K	R	G	K	R	N	N	F	K	R	A	K	Y		
83	T	F	F	Y	G	G	C	L	G	N	R	N	N	F	K	T	E	E	Y		
84	T	F	F	Y	G	C	G	R	G	K	R	N	N	F	K	T	E	E	Y		
85	T	F	F	Y	G	G	R	C	G	K	R	N	N	F	K	T	E	E	Y		
86	T	F	F	Y	G	G	C	L	G	N	G	N	N	F	D	T	E	E	E		
87	T	F	Q	Y	G	G	C	R	G	K	R	N	N	F	K	T	E	E	Y		
88	Y	N	K	E	F	G	T	F	N	T	K	G	C	E	R	G	Y	R	F		
89	R	F	K	Y	G	G	C	L	G	N	M	N	N	F	E	T	L	E	E		
90	R	F	K	Y	G	G	C	L	G	N	K	N	N	F	L	R	L	K	Y		
91	R	F	K	Y	G	G	C	L	G	N	K	N	N	Y	L	R	L	K	Y		
92	K	T	K	R	K	R	K	K	Q	R	V	K	I	A	Y	E	E	I	F	K	N	Y
93	K	T	K	R	K	R	K	K	Q	R	V	K	I	A	Y						
94	R	G	G	R	L	S	Y	S	R	R	F	S	T	S	T	G	R				
95	R	R	L	S	Y	S	R	R	R	F											
96	R	Q	I	K	I	W	F	Q	N	R	R	M	K	W	K	K					
97	T	F	F	Y	G	G	S	R	G	K	R	N	N	F	K	T	E	E	Y		
98	M	R	P	D	F	C	L	E	P	P	Y	T	G	P	C	V	A	R	I		
	I	R	Y	F	Y	N	A	K	A	G	L	C	Q	T	F	V	Y	G	G		
	C	R	A	K	R	N	N	F	K	S	A	E	D	C	M	R	T	C	G	G	A
99	T	F	F	Y	G	G	C	R	G	K	R	N	N	F	K	T	K	E	Y		
100	R	F	K	Y	G	G	C	L	G	N	K	N	N	Y	L	R	L	K	Y		

TABLE 1

101	T	F	F	Y	G	G	C	R	A	K	R	N	N	F	K	R	A	K	Y		
102	N	A	K	A	G	L	C	Q	T	F	V	Y	G	G	C	L	A	K	R	N	N	F
	E	S	A	E	D	C	M	R	T	C	G	G	A								
103	Y	G	G	C	R	A	K	R	N	N	F	K	S	A	E	D	C	M	R	T	C	G
	G	A																				
104	G	L	C	Q	T	F	V	Y	G	G	C	R	A	K	R	N	N	F	K	S	A	E
105	L	C	Q	T	F	V	Y	G	G	C	E	A	K	R	N	N	F	K	S	A		
107	T	F	F	Y	G	G	S	R	G	K	R	N	N	F	K	T	E	E	Y			
108	R	F	F	Y	G	G	S	R	G	K	R	N	N	F	K	T	E	E	Y			
109	R	F	F	Y	G	G	S	R	G	K	R	N	N	F	K	T	E	E	Y			
110	R	F	F	Y	G	G	S	R	G	K	R	N	N	F	R	T	E	E	Y			
111	T	F	F	Y	G	G	S	R	G	K	R	N	N	F	R	T	E	E	Y			
112	T	F	F	Y	G	G	S	R	G	R	R	N	N	F	R	T	E	E	Y			

Peptide no. 5 includes the sequence of SEQ ID NO:5 and is amidated at its C-terminus (see for example Fig. 1)

Peptide No. 67 includes the sequence of SEQ ID NO:67 and is amidated at its C-terminus (see for example Fig. 1)

Peptide No. 76 includes the sequence of SEQ ID NO:76 and is amidated at its C-terminus (see for example Fig. 1).

Peptide no. 91 includes the sequence of SEQ ID NO:91 and is amidated at its C-terminus (see for example Fig. 1).

Peptide No. 107 includes the sequence of SEQ ID NO:97 and is acetylated at its N-terminus.

Peptide No. 109 includes the sequence of SEQ ID NO:109 and is acetylated at its N-terminus.

Peptide No. 110 includes the sequence of SEQ ID NO:110 and is acetylated at its N-terminus.

The amine groups of Angiopep-1 (SEQ ID NO:67) and Angiopep-2 (SEQ ID NO:97) have been used as sites for conjugation of agents. To study the role of amine groups in conjugation and their impact in the overall transport capacity of these vectors, new vectors, based on the Angiopep-1 and Angiopep-2 sequence, were designed with variable reactive amine groups and variable overall charge. These polypeptides are shown in Table 2.

Table 2: Vectors with variable amine group targets

Polypeptide Name	Polypeptide Sequences	Reactive amines (positions)	Charge	SEQ ID No.
Angiopep-3*	Ac[1]-TFFYGGSRGKRNNFKTEEY	2 (10,15)	+1	107
Angiopep-4b	**R**FFYGGSRGKRNNFKTEEY	3 (1,10,15)	+3	108
Angiopep-4a	Ac[1]-**R**FFYGGSRGKRNNFKTEEY	2 (10,15)	+2	109
Angiopep-5	Ac[1]-**R**FFYGGSRGKRNNF**R**TEEY	1 (10)	+2	110
Angiopep-6	TFFYGGSRGKRNNF**R**TEEY	2 (1,10)	+2	111
Angiopep-7	TFFYGGSRG**R**RNNF**R**TEEY	1 (1)	+2	112

*Angiopep-3 is an acetylated form of Angiopep-2.
[1]Ac represents acetylation.

Modified polypeptides

The invention also includes a polypeptide having a modification of an amino acid sequence described herein (e.g., polypeptide having a sequence described in any one of SEQ ID NOS:1-105 and 107-112 such as Angiopep-3, -4a, -4b, -5, -6, or -7). In certain embodiments, the modification does not destroy significantly a desired biological activity. In some embodiments, the modification may cause a reduction in biological activity (e.g., by at least 5%, 10%, 20%, 25%, 35%, 50%, 60%, 70%, 75%, 80%, 90%, or 95%). In other embodiments, the modification has no effect on the biological activity or may increase (e.g., by at least 5%, 10%, 25%, 50%, 100%, 200%, 500%, or 1,000%) the biological activity of the original polypeptide. The modified polypeptide may have or may optimize one or more of the characteristics of a polypeptide of the invention which, in some instance might be needed or desirable. Such characteristics include in vivo stability, bioavailability, toxicity, immunological activity, or immunological identity.

Polypeptides of the invention may include amino acids or sequences modified either by natural processes, such as posttranslational processing, or by chemical modification techniques known in the art. Modifications may occur anywhere in a polypeptide including the polypeptide backbone, the amino acid

side-chains and the amino- or carboxy-terminus. The same type of modification may be present in the same or varying degrees at several sites in a given polypeptide, and a polypeptide may contain more than one type of modification. Polypeptides may be branched as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic polypeptides may result from posttranslational natural processes or may be made synthetically. Other modifications include pegylation, acetylation, acylation, addition of acetomidomethyl (Acm) group, ADP-ribosylation, alkylation, amidation, biotinylation, carbamoylation, carboxyethylation, esterification, covalent attachment to fiavin, covalent attachment to a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of drug, covalent attachment of a marker (e.g., fluorescent or radioactive), covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent crosslinks, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation and ubiquitination.

A modified polypeptide may further include an amino acid insertion, deletion, or substitution, either conservative or non-conservative (e.g., D-amino acids, desamino acids) in the polypeptide sequence (e.g., where such changes do not substantially alter the biological activity of the polypeptide).

Substitutions may be conservative (i.e., wherein a residue is replaced by another of the same general type or group) or non-conservative (i.e., wherein a residue is replaced by an amino acid of another type). In addition, a non-naturally occurring amino acid may substituted for a naturally occurring amino acid (i.e., non-naturally occurring conservative amino acid substitution or a non-naturally occurring non-conservative amino acid substitution).

Polypeptides made synthetically may include substitutions of amino acids not naturally encoded by DNA (e.g., non-naturally occurring or unnatural amino acid). Examples of non-naturally occurring amino acids include D-amino acids, an amino acid having an acetylaminomethyl group attached to a sulfur atom of a cysteine, a pegylated amino acid, the omega amino acids of the formula $NH_2(CH_2)_nCOOH$ wherein n is 2-6, neutral nonpolar amino acids, such as sarcosine, t-butyl alanine, t-butyl glycine, N-methyl isoleucine, and norleucine. Phenylglycine may substitute for Trp, Tyr, or Phe; citrulline and methionine sulfoxide are neutral nonpolar, cysteic acid is acidic, and ornithine is basic. Proline may be substituted with hydroxyproline and retain the conformation conferring properties.

Analogues may be generated by substitutional mutagenesis and retain the biological activity of the original polypeptide. Examples of substitutions identified as "conservative substitutions" are shown in Table 3. If such substitutions result in a change not desired, then other type of substitutions, denominated "exemplary substitutions" in Table 3, or as further described herein in reference to amino acid classes, are introduced and the products screened.

Substantial modifications in function or immunological identity are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation. (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, methionine (Met), Alanine (Ala), Valine (Val), Leucine (Leu), Isoleucine (Ile), Histidine (His), Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine (Phe),

(2) neutral hydrophilic: Cysteine (Cys), Serine (Ser), Threonine (Thr)

(3) acidic/negatively charged: Aspartic acid (Asp), Glutamic acid (Glu)

(4) basic: Asparagine (Asn), Glutamine (Gln), Histidine (His), Lysine (Lys), Arginine (Arg)

(5) residues that influence chain orientation: Glycine (Gly), Proline (Pro);

(6) aromatic: Tryptophan (Trp), Tyrosine (Tyr), Phenylalanine (Phe), Histidine (His),

(7) polar: Ser, Thr, Asn, Gln

(8) basic positively charged: Arg, Lys, His, and;

(9) charged: Asp, Glu, Arg, Lys, His

Other conservative amino acid substitutions are listed in Table 3.

Table 3 Amino acid substitution

Original residue	Exemplary substitution	Conservative substitution
Ala (A)	Val, Leu, Ile	Val
Arg (R)	Lys, Gln, Asn	Lys
Asn (N)	Gln, His, Lys, Arg	Gln
Asp (D)	Glu	Glu
Cys (C)	Ser	Ser
Gln (Q)	Asn	Asn
Glu (E)	Asp	Asp
Gly (G)	Pro	Pro
His (H)	Asn, Gln, Lys, Arg	Arg
Ile (I)	Leu, Val, Met, Ala, Phe, norleucine	Leu
Leu (L)	Norleucine, Ile, Val, Met, Ala, Phe	Ile
Lys (K)	Arg, Gln, Asn	Arg
Met (M)	Leu, Phe, Ile	Leu
Phe (F)	Leu, Val, Ile, Ala	Leu
Pro (P)	Gly	Gly
Ser (S)	Thr	Thr
Thr (T)	Ser	Ser
Trp (W)	Tyr	Tyr
Tyr (Y)	Trp, Phe, Thr, Ser	Phe
Val (V)	Ile, Leu, Met, Phe, Ala, norleucine	Leu

Additional analogues

The polypeptides and conjugates of the invention may include polypeptide analogs of aprotinin known in the art. For example, U.S. Patent No. 5,807,980 describes Bovine Pancreatic Trypsin Inhibitor (aprotinin)-derived inhibitors as well as a method for their preparation and therapeutic use, including the polypeptide of SEQ ID NO:102. These polypeptides have been used for the treatment of a condition characterized by an abnormal appearance or amount of tissue factor and/or factor VIIIa such as abnormal thrombosis. U.S. Patent No. 5,780,265 describes serine protease inhibitors capable of inhibiting plasma kallikrein, including SEQ ID NO:103. U.S. Patent No. 5,118,668 describes Bovine Pancreatic Trypsin Inhibitor variants, including SEQ ID NO:105. The aprotinin amino acid sequence (SEQ ID NO:98), the Angiopep-1 amino acid sequence (SEQ ID NO:67), and SEQ ID NO:104, as well as some sequences of biologically active analogs may be found in International Application Publication No. WO 2004/060403.

An exemplary nucleotide sequence encoding an aprotinin analogue is illustrated in SEQ ID NO:106 (atgagaccag atttctgcct cgagccgccg tacactgggc cctgcaaagc tcgtatcatc cgttacttct acaatgcaaa ggcaggcctg tgtcagacct tcgtatacgg cggctgcaga gctaagcgta acaacttcaa atccgcggaa gactgcatgc gtacttgcgg tggtgcttag; Genbank accession No. X04666). This sequence encodes a lysine at position 16 instead of a valine, as found in SEQ ID NO:98. A mutation in the nucleotide sequence of SEQ ID NO:106 may be introduced by methods known in the art to change the produce the polypeptide of SEQ ID NO:98 having a valine in position 16. Additional mutations or fragments may be obtained using any technique known in the art.

Other examples of aprotinin analogs may be found by performing a protein BLAST (Genebank: www.ncbi.nlm.nih.gov/BLAST/) using the synthetic aprotinin sequence (or portion thereof) disclosed in International Application No. PCT/CA2004/000011. Exemplary aprotinin analogs are found under accession Nos. CAA37967 (GI:58005) and 1405218C (GI:3604747).

Preparation of polypeptide derivatives and peptidomimetics

In addition to polypeptides consisting only of naturally occurring amino acids, peptidomimetics or polypeptide analogs are also encompassed by the present invention. Polypeptide analogs are commonly used in the pharmaceutical industry as non-polypeptide drugs with properties analogous to those of the template polypeptide. The non-polypeptide compounds are termed "polypeptide mimetics" or peptidomimetics (Fauchere et al., *Infect. Immun.* 54:283-287,1986; Evans et al., *J. Med. Chem.* 30:1229-1239, 1987). Polypeptide mimetics that are structurally related to therapeutically useful polypeptides may be used to produce an equivalent or enhanced therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to the paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity) such as naturally-occurring receptor-binding polypeptides, but have one or more peptide linkages optionally replaced by linkages such as $-CH_2NH-$, $-CH_2S-$, $-CH_2-CH_2-$, $-CH=CH-$ (cis and trans), $-CH_2SO-$, $-CH(OH)CH_2-$, $-COCH_2-$ etc., by methods well known in the art (Spatola, *Peptide Backbone Modifications, Vega Data,* 1(3):267, 1983); Spatola et al. (*Life Sci.* 38:1243-1249, 1986); Hudson et al. (*Int. J. Pept. Res.* 14:177-185, 1979); and Weinstein. B., 1983, Chemistry and Biochemistry, of Amino Acids, Peptides and Proteins, Weinstein eds, Marcel Dekker, New-York). Such polypeptide mimetics may have significant advantages over naturally-occurring polypeptides including more economical production, greater chemical stability, enhanced pharmacological properties (e.g., half-life, absorption, potency, efficiency), reduced antigenicity and others.

While the polypeptides of the invention may be effective in entering particular cell types (e.g., those described herein), their effectiveness may be reduced by the presence of proteases. Serum proteases have specific substrate requirements. The substrate must have both L-amino acids and peptide bonds for cleavage. Furthermore, exopeptidases, which represent the most prominent component of the protease activity in serum, usually act on the first peptide

bond of the polypeptide and require a free N-terminus (Powell et al., *Pharm. Res.* 10:1268-1273, 1993). In light of this, it is often advantageous to use modified versions of polypeptides. The modified polypeptides retain the structural characteristics of the original L-amino acid polypeptides that confer biological activity with regard to IGF-1, but are advantageously not readily susceptible to cleavage by protease and/or exopeptidases.

Systematic substitution of one or more amino acids of a consensus sequence with D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more stable polypeptides. Thus, a polypeptide derivative or peptidomimetic of the present invention may be all L, all D or mixed D, L polypeptide. The presence of an N-terminal or C-terminal D-amino acid increases the *in vivo* stability of a polypeptide because peptidases cannot utilize a D-amino acid as a substrate (Powell et al., *Pharm. Res.* 10:1268-1273, 1993). Reverse-D polypeptides are polypeptides containing D-amino acids, arranged in a reverse sequence relative to a polypeptide containing L-amino acids. Thus, the C-terminal residue of an L-amino acid polypeptide becomes N-terminal for the D-amino acid polypeptide, and so forth. Reverse D-polypeptides retain the same tertiary conformation and therefore the same activity, as the L-amino acid polypeptides, but are more stable to enzymatic degradation *in vitro* and *in vivo*, and thus have greater therapeutic efficacy than the original polypeptide (Brady and Dodson, *Nature* 368:692-693, 1994; Jameson et al., *Nature* 368:744-746, 1994). In addition to reverse-D-polypeptides, constrained polypeptides comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods well known in the art (Rizo and Gierasch, *Ann. Rev. Biochem.* 61:387-418, 1992). For example, constrained polypeptides may be generated by adding cysteine residues capable of forming disulfide bridges and, thereby, resulting in a cyclic polypeptide. Cyclic polypeptides have no free N- or C-termini. Accordingly, they are not susceptible to proteolysis by exopeptidases, although they are, of course, susceptible to endopeptidases,

which do not cleave at peptide termini. The amino acid sequences of the polypeptides with N-terminal or C-terminal D-amino acids and of the cyclic polypeptides are usually identical to the sequences of the polypeptides to which they correspond, except for the presence of N-terminal or C-terminal D-amino acid residue, or their circular structure, respectively.

A cyclic derivative containing an intramolecular disulfide bond may be prepared by conventional solid phase synthesis while incorporating suitable S-protected cysteine or homocysteine residues at the positions selected for cyclization such as the amino and carboxy termini (Sah et al., *J. Pharm. Pharmacol.* 48:197, 1996). Following completion of the chain assembly, cyclization can be performed either (1) by selective removal of the S-protecting group with a consequent on-support oxidation of the corresponding two free SH-functions, to form a S-S bonds, followed by conventional removal of the product from the support and appropriate purification procedure or (2) by removal of the polypeptide from the support along with complete side chain de-protection, followed by oxidation of the free SH-functions in highly dilute aqueous solution.

The cyclic derivative containing an intramolecular amide bond may be prepared by conventional solid phase synthesis while incorporating suitable amino and carboxyl side chain protected amino acid derivatives, at the position selected for cyclization. The cyclic derivatives containing intramolecular -S-alkyl bonds can be prepared by conventional solid phase chemistry while incorporating an amino acid residue with a suitable amino-protected side chain, and a suitable S-protected cysteine or homocysteine residue at the position selected for cyclization.

Another effective approach to confer resistance to peptidases acting on the N-terminal or C-terminal residues of a polypeptide is to add chemical groups at the polypeptide termini, such that the modified polypeptide is no longer a substrate for the peptidase. One such chemical modification is glycosylation of the polypeptides at either or both termini. Certain chemical

modifications, in particular N-terminal glycosylation, have been shown to increase the stability of polypeptides in human serum (Powell et al., *Pharm. Res.* 10:1268-1273, 1993). Other chemical modifications which enhance serum stability include, but are not limited to, the addition of an N-terminal alkyl group, consisting of a lower alkyl of from one to twenty carbons, such as an acetyl group, and/or the addition of a C-terminal amide or substituted amide group. In particular, the present invention includes modified polypeptides consisting of polypeptides bearing an N-terminal acetyl group and/or a C-terminal amide group.

Also included by the present invention are other types of polypeptide derivatives containing additional chemical moieties not normally part of the polypeptide, provided that the derivative retains the desired functional activity of the polypeptide. Examples of such derivatives include (1) N-acyl derivatives of the amino terminal or of another free amino group, wherein the acyl group may be an alkanoyl group (e.g., acetyl, hexanoyl, octanoyl) an aroyl group (e.g., benzoyl) or a blocking group such as F-moc (fluorenylmethyl–O–CO–); (2) esters of the carboxy terminal or of another free carboxy or hydroxyl group; (3) amide of the carboxy-terminal or of another free carboxyl group produced by reaction with ammonia or with a suitable amine; (4) phosphorylated derivatives; (5) derivatives conjugated to an antibody or other biological ligand and other types of derivatives.

Longer polypeptide sequences which result from the addition of additional amino acid residues to the polypeptides of the invention are also encompassed in the present invention. Such longer polypeptide sequences would be expected to have the same biological activity (e.g., entering particular cell types) as the polypeptides described above. While polypeptides having a substantial number of additional amino acids are not excluded, it is recognized that some large polypeptides may assume a configuration that masks the effective sequence, thereby preventing binding to a target (e.g., a member of the LRP receptor family such as LRP or LRP2). These derivatives could act as

competitive antagonists. Thus, while the present invention encompasses polypeptides or derivatives of the polypeptides described herein having an extension, desirably the extension does not destroy the cell targeting activity of the polypeptide or derivative.

Other derivatives included in the present invention are dual polypeptides consisting of two of the same, or two different polypeptides of the present invention covalently linked to one another either directly or through a spacer, such as by a short stretch of alanine residues or by a putative site for proteolysis (e.g., by cathepsin, see e.g., U.S. Patent No. 5,126,249 and European Patent No. 495 049). Multimers of the polypeptides of the present invention consist of polymer of molecules formed from the same or different polypeptides or derivatives thereof.

The present invention also encompasses polypeptide derivatives that are chimeric or fusion proteins containing a polypeptide described herein, or fragment thereof, linked at its amino- or carboxy-terminal end, or both, to an amino acid sequence of a different protein. Such a chimeric or fusion protein may be produced by recombinant expression of a nucleic acid encoding the protein. For example, a chimeric or fusion protein may contain at least 6 amino acids of a polypeptide of the present invention and desirably has a functional activity equivalent or greater than a polypeptide of the invention.

Polypeptide derivatives of the present invention can be made by altering the amino acid sequences by substitution, addition, or deletion or an amino acid residue to provide a functionally equivalent molecule, or functionally enhanced or diminished molecule, as desired. The derivative of the present invention include, but are not limited to, those containing, as primary amino acid sequence, all or part of the amino acid sequence of the polypeptides described herein (e.g., any one of SEQ ID NOS:1-105 and 107-112) including altered sequences containing substitutions of functionally equivalent amino acid residues. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a

functional equivalent, resulting in a silent alteration. Substitution for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the positively charged (basic) amino acids include, arginine, lysine and histidine. The nonpolar (hydrophobic) amino acids include, leucine, isoleucine, alanine, phenylalanine, valine, proline, tryptophan and methionine. The uncharged polar amino acids include serine, threonine, cysteine, tyrosine, asparagine and glutamine. The negatively charged (acid) amino acids include glutamic acid and aspartic acid. The amino acid glycine may be included in either the nonpolar amino acid family or the uncharged (neutral) polar amino acid family. Substitutions made within a family of amino acids are generally understood to be conservative substitutions.

Assays to identify peptidomimetics

As described above, non-peptidyl compounds generated to replicate the backbone geometry and pharmacophore display (peptidomimetics) of the polypeptides identified by the methods of the present invention often possess attributes of greater metabolic stability, higher potency, longer duration of action and better bioavailability.

The peptidomimetics compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to polypeptide libraries, while the other four approaches are applicable to polypeptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Des.* 12:145, 1997). Examples of methods for the synthesis of molecular libraries can be found in the art, for example, in: DeWitt et al. (*Proc. Natl. Acad. Sci. USA* 90:6909,

1993); Erb et al. (*Proc. Natl. Acad. Sci. USA* 91:11422, 1994); Zuckermann et al., *J. Med. Chem.* 37:2678, 1994); Cho et al. (*Science* 261:1303, 1993); Carell et al. (*Angew. Chem, Int. Ed. Engl.* 33:2059, 1994 and ibid 2061); and in Gallop et al. (*Med. Chem.* 37:1233, 1994). Libraries of compounds may be presented in solution (e.g., Houghten, *Biotechniques* 13:412-421, 1992) or on beads (Lam, *Nature* 354:82-84, 1991), chips (Fodor, *Nature* 364:555-556, 1993), bacteria or spores (U.S. Patent No. 5,223,409), plasmids (Cull et al., *Proc. Natl. Acad. Sci. USA* 89:1865-1869, 1992) or on phage (Scott and Smith, *Science* 249:386-390, 1990), or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

Once a polypeptide of the present invention is identified, it may be isolated and purified by any number of standard methods including, but not limited to, differential solubility (e.g., precipitation), centrifugation, chromatography (e.g., affinity, ion exchange, size exclusion, and the like) or by any other standard techniques used for the purification of polypeptides, peptidomimetics or proteins. The functional properties of an identified polypeptide of interest may be evaluated using any functional assay known in the art. Desirably, assays for evaluating downstream receptor function in intracellular signaling are used (e.g., cell proliferation).

For example, the peptidomimetics compounds of the present invention may be obtained using the following three-phase process: (1) scanning the polypeptides of the present invention to identify regions of secondary structure necessary for targeting the particular cell types described herein; (2) using conformationally constrained dipeptide surrogates to refine the backbone geometry and provide organic platforms corresponding to these surrogates; and (3) using the best organic platforms to display organic pharmocophores in libraries of candidates designed to mimic the desired activity of the native polypeptide. In more detail the three phases are as follows. In phase 1, the lead candidate polypeptides are scanned and their structure abridged to identify the requirements for their activity. A series of polypeptide analogs of the

original are synthesized. In phase 2, the best polypeptide analogs are investigated using the conformationally constrained dipeptide surrogates. Indolizidin-2-one, indolizidin-9-one and quinolizidinone amino acids (I^2aa, I^9aa and Qaa respectively) are used as platforms for studying backbone geometry of the best polypeptide candidates. These and related platforms (reviewed in Halab et al., *Biopolymers* 55:101-122, 2000; and Hanessian et al. *Tetrahedron* 53:12789-12854, 1997) may be introduced at specific regions of the polypeptide to orient the pharmacophores in different directions. Biological evaluation of these analogs identifies improved lead polypeptides that mimic the geometric requirements for activity. In phase 3, the platforms from the most active lead polypeptides are used to display organic surrogates of the pharmacophores responsible for activity of the native polypeptide. The pharmacophores and scaffolds are combined in a parallel synthesis format. Derivation of polypeptides and the above phases can be accomplished by other means using methods known in the art.

Structure function relationships determined from the polypeptides, polypeptide derivatives, peptidomimetics, or other small molecules of the present invention may be used to refine and prepare analogous molecular structures having similar or better properties. Accordingly, the compounds of the present invention also include molecules that share the structure, polarity, charge characteristics, and side chain properties of the polypeptides described herein.

In summary, based on the disclosure herein, those skilled in the art can develop polypeptides and peptidomimetics screening assays which are useful for identifying compounds for targeting an agent to particular cell types (e.g., those described herein). The assays of this invention may be developed for low-throughput, high-throughput, or ultra-high throughput screening formats. Assays of the present invention include assays which are amenable to automation.

Conjugates of the invention

The polypeptides described herein or derivatives thereof may be linked to an agent. For example, the polypeptide (e.g., Angiopep-7) may be attached to a therapeutic agent, a diagnostic agent, or to a label. In certain embodiments, the polypeptide is linked to or labeled with a detectable label, such as a radioimaging agent, for diagnosis of a disease or condition. Examples of these agents include a radioimaging agent-antibody-vector conjugate, where the antibody binds to a disease or condition-specific antigen (e.g., for diagnosis or therapy). Other binding molecules are also contemplated by the invention. In other cases, the polypeptide or derivative is linked to a therapeutic agent, to treat a disease or condition, or may be linked to or labeled with mixtures thereof. The disease or condition may be treated by administering a vector-agent conjugate to an individual under conditions which allow transport of the agent across the BBB or into a particular cell type. Each polypeptide may include at least 1, 2, 3, 4, 5, 6, or 7 agents. In other embodiments, each agent has at least 1, 2, 3, 4, 5, 6 7, 10, 15, 20, or more polypeptides attached thereto. The conjugates of the invention may be able to promote accumulation (e.g., due to increased uptake or reduced removal) of the agent in a particular cell type or tissue such as liver, lung, kidney, spleen or muscle of a subject.

The agent may be releasable from the vector after transport into a particular cell type or across the BBB. The agent can be released, for example, by enzymatic cleavage or other breakage of a chemical bond between the vector and the agent. The released agent may then function in its intended capacity in the absence of the vector.

Therapeutic agents

A therapeutic agent may be any biologically active agent. For example, a therapeutic may be a drug, a medicine, an agent emitting radiation, a cellular toxin (for example, a chemotherapeutic agent), a biologically active fragment thereof, or a mixture thereof to treat a disease (e.g., to killing cancer cells) or it

may be an agent to treat a disease or condition in an individual. A therapeutic agent may be a synthetic product or a product of fungal, bacterial or other microorganism (e.g., mycoplasma or virus), animal, such as reptile, or plant origin. A therapeutic agent and/or biologically active fragment thereof may be an enzymatically active agent and/or fragment thereof, or may act by inhibiting or blocking an important and/or essential cellular pathway or by competing with an important and/or essential naturally occurring cellular component. Other therapeutic agents include antibodies and antibody fragments.

Anticancer agents. Any anticancer agent known in the art may be part of a conjugate of the invention. Cancers of the brain may be treated with a conjugate containing a vector that is efficiently transported across the BBB (e.g., Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, or Angiopep-6). Liver, lung, kidney, or spleen cancers may be treated with an anticancer agent conjugated to a vector that is transported efficiently into the appropriate cell type (e.g., Angiopep-7). Exemplary agents include abarelix, aldesleukin, alemtuzumab, alitretinoin, allopurinol, altretamine, amifostine, anakinra, anastrozole, arsenic trioxide, asparaginase, azacitidine, BCG Live, bevacuzimab, bexarotene, bleomycin, bleomycin, bortezombi, bortezomib, busulfan, busulfan, calusterone, capecitabine, carboplatin, carmustine, celecoxib, cetuximab, chlorambucil, cisplatin, cladribine, clofarabine, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, actinomycin D, dalteparin (e.g., sodium), darbepoetin alfa, dasatinib, daunorubicin, daunomycin, decitabine, denileukin, denileukin diftitox, dexrazoxane, docetaxel, doxorubicin, dromostanolone propionate, eculizumab, epirubicin (e.g., HCl), epoetin alfa, erlotinib, estramustine, etoposide (e.g., phosphate), exemestane, fentanyl (e.g., citrate), filgrastim, floxuridine, fludarabine, fluorouracil, 5-FU, fulvestrant, gefitinib, gemcitabine (e.g., HCl), gemtuzumab ozogamicin, goserelin (e.g., acetate), histrelin (e.g., acetate), hydroxyurea, ibritumomab tiuxetan, idarubicin, ifosfamide, imatinib (e.g., mesylate),

Interferon alfa-2b, irinotecan, lapatinib ditosylate, lenalidomide, letrozole, leucovorin, leuprolide (e.g., acetate), levamisole, lomustine, CCNU, meclorethamine (nitrogen mustard), megestrol, melphalan (L-PAM), mercaptopurine (6-MP), mesna, methotrexate, methoxsalen, mitomycin C, mitotane, mitoxantrone, nandrolone phenpropionate, nelarabine, nofetumomab, oprelvekin, oxaliplatin, paclitaxel, palifermin, pamidronate, panitumumab, pegademase, pegaspargase, pegfilgrastim, peginterferon alfa-2b, pemetrexed (e.g., disodium), pentostatin, pipobroman, plicamycin (mithramycin), porfimer (e.g., sodium), procarbazine, quinacrine, rasburicase, rituximab, sargramostim, sorafenib, streptozocin, sunitinib (e.g., maleate), talc, tamoxifen, temozolomide, teniposide (VM-26), testolactone, thalidomide, thioguanine (6-TG), thiotepa, thiotepa, thiotepa, topotecan (e.g., hcl), toremifene, Tositumomab/I-131 (tositumomab), trastuzumab, trastuzumab, tretinoin (ATRA), uracil mustard, valrubicin, vinblastine, vincristine, vinorelbine, vorinostat, zoledronate, and zoledronic acid. Exemplary derivatives of paclitaxel are described in U.S. Patent No. 6,911,549, the entire contents of which are hereby incorporated by reference.

Detectable labels

For the purpose of detection or diagnosis, the conjugate of the invention may be labeled. Detectable labels, or markers, may be a radiolabel, a fluorescent label, a nuclear magnetic resonance active label, a luminescent label, a chromophore label, a positron emitting isotope for PET scanner, chemiluminescence label, or an enzymatic label. Exemplary radioimaging agents emitting radiation (detectable radiolabels) include indium-111, technitium-99, or low dose iodine-131. Gamma and beta emitting radionuclides include ^{67}Cu, ^{67}Ga, ^{90}Y, ^{111}In, ^{99m}Tc, and ^{201}Tl). Positron emitting radionuclides include ^{18}F, ^{55}Co, ^{60}Cu, ^{62}Cu, ^{64}Cu, ^{66}Ga, ^{68}Ga, ^{82}Rb, and ^{86}Y. Fluorescent labels include Cy5.5, green fluorescent protein (GFP), fluorescein, and rhodamine. Chemiluminescence labels include luciferase and β-

galactosidase. Enzymatic labels include peroxidase and phosphatase. A histag may also be a detectable label. For example, conjugates may include a vector moiety and an antibody moiety (antibody or antibody fragment), which may further include a label. In this case, the label may be attached to either the vector or to the antibody.

Antibodies

Antibodies may also be conjugated to the polypeptides of the invention by any means known in the art (e.g., using the conjugation strategies described herein). Any diagnostic or therapeutic antibody may be conjugated to one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) vectors of the invention. In addition, antibody fragments (e.g., capable of binding to an antigen) may also be conjugated to the vectors of the invention. Antibody fragments include the Fab and Fc regions, heavy chain, and light chain of an antibody (e.g., of any antibody described herein). Exemplary antibodies for use in diagnosis and therapy of cancer include ABX-EGF (Panitimumab), OvaRex (Oregovemab), Theragyn (pemtumomabytrrium-90), Therex, Bivatuzumab, Panorex (Edrecolomab), ReoPro (Abciximab), Bexxar (Tositumomab), MAb, idiotypic 105AD7, Anti-EpCAM (Catumaxomab), MAb lung cancer (from Cytoclonal), Herceptin (Trastuzumab), Rituxan (Rituximab), Avastin (Bevacizumab), AMD Fab (Ranibizumab), E-26 (2^{nd} gen. IgE) (Omalizumab), Zevalin (Rituxan + yttrium-90) (Ibritumomab tiuxetan), Cetuximab, BEC2 (Mitumomab), IMC-1C11, nuC242-DM1, LymphoCide (Epratuzumab), LymphoCide Y-90, CEA-Cide (Labetuzumab), CEA-Cide Y-90, CEA-Scan (Tc-99m-labeled arcitumomab), LeukoScan (Tc-99m-labeled sulesomab), LymphoScan (Tc-99m-labeled bectumomab), AFP-Scan (Tc-99m-labeled), HumaRAD-HN (+ yttrium-90), HumaSPECT (Votumumab), MDX-101 (CTLA-4), MDX-210 (her-2 overexpression), MDX-210/MAK, Vitaxin, MAb 425, IS-IL-2, Campath (alemtuzumab), CD20 streptavidin, Avidicin, (albumin + NRLU13), Oncolym (+ iodine-131) Cotara (+ iodine-131), C215 (+ staphylococcal enterotoxin,

MAb lung/kidney cancer (from Pharmacia Corp.), nacolomab tafenatox (C242 staphylococcal enterotoxin), Nuvion (Visilizumab), SMART M195, SMART 1D10, CEAVac, TriGem, TriAb, NovoMAb-G2 radiolabeled, Monopharm C, GlioMAb-H (+ gelonin toxin), Rituxan (Rituximab), and ING-1. Additional therapeutic antibodies include 5G1.1 (Ecluizumab), 5G1.1-SC (Pexelizumab), ABX-CBL (Gavilimomab), ABX-IL8, Antegren (Natalizumab), Anti-CD11a (Efalizumab), Anti-CD18 (from Genetech), Anti-LFA1, Antova, BTI-322, CDP571, CDP850, Corsevin M, D2E7 (Adalimumab), Humira (Adalimumab), Hu23F2G (Rovelizumab), IC14, IDEC-114, IDEC-131, IDEC-151, IDEC-152, Infliximab (Remicade), LDP-01, LDP-02, MAK-195F (Afelimomab), MDX-33, MDX-CD4, MEDI-507 (Siplizumab), OKT4A, OKT3 (Muromonab- CD3), and ReoPro (Abciximab).

Conjugation linkers

The conjugate (e.g., a protein-protein conjugate) may be obtained using any cross-linking (conjugation) reagent or protocol know in the art, many of which are commercially available. Such protocols and reagents include, cross-linkers reactive with amino, carboxyl, sulfhydryl, carbonyl, carbohydrate and/or phenol groups. The amounts, times, and conditions of such protocols can be varied to optimize conjugation. Cross-linking reagents contain at least two reactive groups and are generally divided into homofunctional cross-linkers (containing identical reactive groups) and heterofunctional cross-linkers (containing non-identical reactive groups). The cross-linkers of the invention may be either homobifunctional and/or heterobifunctional. Furthermore the cross-linker may incorporate a 'spacer' between the reactive moieties, or the two reactive moieties in the cross-linker may be directly linked. Bonds may include ester bonds.

Exemplary linkers include BS^3 [Bis(sulfosuccinimidyl)suberate], NHS/EDC (N-hydroxysuccinimide and N-ethyl-(dimethylaminopropyl)carbodimide, Sulfo-EMCS ([N-e-Maleimidocaproic

acid]hydrazide), SATA (N-succinimidyl-S-acetylthioacetate), and hydrazide. BS^3 is a homobifunctional N-hydroxysuccinimide ester that targets accessible primary amines. A conjugation scheme is exemplified in Fig. 2. NHS/EDC allows for the conjugation of primary amine groups with carboxyl groups. Sulfo-EMCS are heterobifunctional reactive groups (maleimide and NHS-ester) that are reactive toward sulfhydryl and amino groups. Amine coupling using sulfo-NHS/EDC activation may be used to cross-link therapeutic antibodies with the polypeptides of the invention, as exemplified in Figs. 3 and 4. This is a fast, simple and reproducible coupling technique. The resulting conjugate is stable and retains the biological activity of the antibody. Moreover, it has a high conjugation capacity that can be reliably controlled and a low non-specific interaction during coupling procedures. SATA is reactive towards amines and adds protected sulfhydryls groups. The NHS-ester reacts with primary amines to form stable amide bonds. Sulfhydryl groups may be deprotected using hydroxylamine. This conjugation method is exemplified in Fig. 5. Hydrazide can be used to link carboxyl groups to primary amines, as shown in Fig. 6, and may therefore be useful for linking glycoproteins. Additional exemplary linkers are illustrated in Fig. 7.

Small molecules such as therapeutic agents can be conjugated to the polypeptides of the invention. The exemplary small molecule, paclitaxel, has two strategic positions (position C2' and C7) useful for conjugation. Conjugation of a vector or vector of the invention to paclitaxel can be performed as follows (Fig. 8). Briefly, paclitaxel is reacted with anhydride succinic pyridine for three hours at room temperature to attach a succinyl group in position 2'. The 2'-succinyl paclitaxel has a cleavable ester bond in position 2' can simply release succinic acid. This cleavable ester bond can be further used for various modifications with linkers, if desired. The resulting 2'-O-succinyl-paclitaxel is then reacted with EDC/NHS in DMSO for nine hours at room temperature, followed by the addition of the vector or vector in Ringer/DMSO for an additional reaction time of four hours at room

temperature. The reaction of conjugation depicted in Fig. 8 is monitored by HPLC. Each intermediate, such as paclitaxel, 2'-O-succinyl-paclitaxel and 2'-O-NHS-succinyl-paclitaxel, is purified and validated using different approaches such as HPLC, thin liquid chromatography, NMR (^{13}C or ^{1}H exchange), melting point, or mass spectrometry. The final conjugate is analyzed by mass spectrometry and SDS-polyacrylamide gel electrophoresis. This allows determining the number of paclitaxel molecules conjugated on each vector.

Conjugate activities

By conjugating and agent to a vector described herein, desirable properties, such as altered pharmacokinetics, altered tissue distribution (e.g., increased delivery to particular tissues or cell types such as liver, brain, lung, spleen, or kidney) may be achieved. Briefly, we have identified vectors that efficiently transport agents across the BBB (e.g., Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, and Angiopep-6). Like Angiopep-2, these vectors may also be capable of targeting agents to other cell types or tissues (e.g., liver, lung, kidney, spleen, or muscle). We have also identified a vector, Angiopep-7, which is not efficiently transported across the BBB, but is transported to particular tissues (e.g., liver, lung, kidney, spleen, or muscle). Accordingly, vectors with this activity may be useful where transport across the BBB is not desired.

Because the conjugates of the invention transport agents to specific tissues, conjugated agents may result in lower toxicity (e.g., fewer side effects), higher efficacy (e.g., because the agent is concentrated into a target tissue due to increased uptake or decreased efflux from the tissue or cells or because the agent has greater stability when conjugated), or a combination thereof. Such activities are described below and in International Publication No. WO 2007/009229, hereby incorporated by reference.

In some cases, conjugation of an agent to a vector allows the agent to escape the action of P-glycoprotein (P-gp), an efflux pump capable of expelling certain agents from a cell. By decreasing the ability of P-gp to expel an agent from a cell, the potency of that agent in a cell can be increased. These conjugates can thus actively inhibit cancer cell proliferation. Moreover, results obtained for in vivo tumor growth indicate that the vectors of the invention may target the receptor LRP. Also, conjugation may modify the pharmacokinetics or biodistribution of the unconjugated agent.

Taken together, conjugates can be used against primary tumors including breast, lung, and skin cancers as well as metastasis originating from primary tumors.

P-glycoprotein bypass

Because the resistance towards various chemotherapeutic agents such as vincristine, etoposide, and doxorubicin is mediated through P-gp (MDR1) overexpression (Fig. 9), bypassing this efflux pump may potentiate the action of these drugs on various cancer types. By conjugating a vector to such an agent, it is now possible to decrease P-gp mediated efflux of the agent. These conjugates may be useful for increasing the potency of drugs associated with resistance mediated by P-gp. As exemplified below, the vectors described herein were tested for their ability to avoid expulsion by P-gp.

LRP mediated uptake

Based on our previous work (see International Patent Application No. PCT/CA2004/00011, hereby incorporated by reference), receptor-associated protein (RAP) inhibited transcytosis of aprotinin in an in vitro model of the blood brain barrier. LRP is expressed on a variety of cancer cells (see, e.g., International Publication No. WO 2007/009229). We therefore proposed that the low-density lipoprotein related receptor (LRP) is involved in the penetration of aprotinin into the brain. Similar inhibition of Angiopep-1 and

Angiopep-2 transport across an in vitro model of the blood-brain barrier was also obtained (data not shown) suggesting that transcytosis of these polypeptide across brain endothelial cell also involved LRP as well. On this basis, we believe that LRP may be involved, generally, in uptake of the aprotinin related polypeptides described herein.

LRP is a heterodimeric membrane receptor of 600 kDa composed of two subunits; the subunit-α (515 kDa) and the subunit-β (85 kDa). Because LRP may be involved in the transport of a vector as described herein, the conjugates of the invention may target cells and tumors expressing this receptor. Certain cancer cells, for example, express a member of the LRP (e.g., LRP or LRP2).

Methods of treatment

The invention also features methods of treatment using the polypeptide conjugates described herein. Conjugates that are efficiently transported across the BBB (e.g., Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, and Angiopep-6) may be used to treat any brain or central nervous system disease. These conjugates are also efficiently transported to the liver, lung, kidney, spleen or muscle and therefore may also be used, in conjunction with an appropriate therapeutic agent, to treat a disease associated with these tissues (e.g., a cancer). Because Angiopep-7 is not efficiently transported to the brain, but is transported efficiently to tissues and cells such as liver, lung, kidney, spleen and muscle, Angiopep-7 may be especially well suited as a vector treatment of diseases associated with these tissues when targeting the agent to the brain is not desired.

Liver diseases include amebic liver abscess, cirrhosis, disseminated coccidioidomycosis; drug-induced cholestasis, hemochromatosis, hepatitis A, hepatitis B, hepatitis C, hepatitis D, hepatocellular carcinoma, liver cancer, liver disease due to alcohol, primary biliary cirrhosis, pyogenic liver abscess, Reye' syndrome, sclerosing cholangitis, and Wilson's disease. Amebic liver abscess may be treated by administration of a vector conjugated to

metronidazole. Hepatitis B may be treated, for example, by administration of vector conjugated to interferon-alpha, lamivudine, adefovir dipivoxil, entecavir, or other antiviral agent. Hepatitis C may be treated, for example, by administration of a vector conjugated to pegylated interferon or ribavirin, or a combination thereof.

Lung diseases include lung cancers such as small cell carcinoma (e.g., oat cell cancer), mixed small cell/large cell carcinoma, combined small cell carcinoma, and metastatic tumors. Metastatic tumors can originate from cancer of any tissue, including breast cancer, colon cancer, prostate cancer, sarcoma, bladder cancer, neuroblastoma, and Wilm's tumor. Spleen diseases include cancers such as lymphoma, non-Hodgkin's lymphoma, and certain T-cell lymphomas.

Additional exemplary cancers that may be treated using a conjugate or composition of the invention include hepatocellular carcinoma, breast cancer, cancers of the head and neck including various lymphomas such as mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, cancers of the retina, cancers of the esophagus, multiple myeloma, ovarian cancer, uterine cancer, melanoma, colorectal cancer, bladder cancer, prostate cancer, lung cancer (including non-small cell lung carcinoma), pancreatic cancer, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adenocarcinoma, parotid adenocarcinoma, endometrial sarcoma, multidrug resistant cancers; and proliferative diseases and conditions, such as neovascularization associated with tumor angiogenesis, macular degeneration (e.g., wet/dry AMD), comeal neovascularization, diabetic retinopathy, neovascular glaucoma, myopic degeneration and other proliferative diseases and conditions such as restenosis and polycystic kidney disease. Brain cancers that may be treated with vector that is transported efficiently across the BBB include astrocytoma, pilocytic astrocytoma, dysembryoplastic neuroepithelial tumor, oligodendrogliomas, ependymoma,

glioblastoma multiforme, mixed gliomas, oligoastrocytomas, medulloblastoma, retinoblastoma, neuroblastoma, germinoma, and teratoma.

A conjugate or composition of the invention may be administered by any means known in the art; e.g., orally, intraarterially, intranasally, intraperitoneally, intravenously, intramuscularly, subcutaneously, transdermally, or *per os* to the subject. The agent may be, for example, an anti-angiogenic compound.

Pharmaceutical compositions

Pharmaceutical compositions of the invention may include a polypeptide or conjugate described herein, in association with a pharmaceutically acceptable carrier. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl, acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts). Solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines). Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or

permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal, oral, vaginal, rectal routes. In one embodiment the pharmaceutical composition is administered parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intradermally, subcutaneously, intraperitonealy, intraventricularly, intracranially, and intratumorally.

Pharmaceutically acceptable carriers further include 0.01-0.1 M or 0.05 M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's orfixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

Other formulations include poly-oxyethylene esters of a fatty acid (e.g., 12-hydroxystearic acid) such as Solutol® HS15. Thus, in some embodiments, a pharmaceutical composition may comprise a) a conjugate described herein, b) Solutol® HS15 and c) an aqueous solution or buffer (e.g., Ringer/Hepes solution at a pH of 5 to 7). The concentration of Solutol® HS15 in the formulation may be at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, or 60% (e.g., 30%) or within any range between any two of these numbers. The concentration of conjugate may be determined based upon the dose required for efficiently treating a subject, or the amount the ester required for solubility of the conjugate being administered. The use of Solutol in a formulation for administration of a Taxol conjugate is described, for example,

in International Publication No. WO 2007/009229, hereby incorporated by reference.

Solid dosage forms for oral use

Formulations for oral use include tablets containing the active ingredient(s) in a mixture with non-toxic pharmaceutically acceptable excipients, and such formulations are known to the skilled artisan (e.g., U.S.P.N.: 5,817,307, 5,824,300, 5,830,456, 5,846,526, 5,882,640, 5,910,304, 6,036,949, 6,036,949, 6,372,218, hereby incorporated by reference). These excipients may be, for example, inert diluents or fillers (e.g., sucrose, sorbitol, sugar, mannitol, microcrystalline cellulose, starches including potato starch, calcium carbonate, sodium chloride, lactose, calcium phosphate, calcium sulfate, or sodium phosphate); granulating and disintegrating agents (e.g., cellulose derivatives including microcrystalline cellulose, starches including potato starch, croscarmellose sodium, alginates, or alginic acid); binding agents (e.g., sucrose, glucose, sorbitol, acacia, alginic acid, sodium alginate, gelatin, starch, pregelatinized starch, microcrystalline cellulose, magnesium aluminum silicate, carboxymethylcellulose sodium, methylcellulose, hydroxypropyl methylcellulose, ethylcellulose, polyvinylpyrrolidone, or polyethylene glycol); and lubricating agents, glidants, and anti-adhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc). Other pharmaceutically acceptable excipients can be colorants, flavoring agents, plasticizers, humectants, buffering agents, and the like.

The tablets may be uncoated or they may be coated by known techniques, optionally to delay disintegration and absorption in the gastrointestinal tract and thereby providing a sustained action over a longer period. The coating may be adapted to release the agent in a predetermined pattern (e.g., in order to achieve a controlled release formulation) or it may be adapted not to release the agent(s) until after passage of the stomach (enteric coating). The coating may be a sugar coating, a film coating (e.g., based on

hydroxypropyl methylcellulose, methylcellulose, methyl hydroxyethylcellulose, hydroxypropylcellulose, carboxymethylcellulose, acrylate copolymers, polyethylene glycols, and/or polyvinylpyrrolidone), or an enteric coating (e.g., based on methacrylic acid copolymer, cellulose acetate phthalate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, shellac, and/or ethylcellulose). Furthermore, a time delay material such as, e.g., glyceryl monostearate or glyceryl distearate, may be employed.

The solid tablet compositions may include a coating adapted to protect the composition from unwanted chemical changes, (e.g., chemical degradation prior to the release of the active substances). The coating may be applied on the solid dosage form in a similar manner as that described in *Encyclopedia of Pharmaceutical Technology*, supra.

The compositions of the invention may be mixed together in the tablet, or may be partitioned. In one example, a first agent is contained on the inside of the tablet, and a second agent is on the outside, such that a substantial portion of the second agent is released prior to the release of the first agent.

Formulations for oral use may also be presented as chewable tablets, or as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent (e.g., potato starch, lactose, microcrystalline cellulose, calcium carbonate, calcium phosphate, or kaolin), or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin, or olive oil. Powders and granulates may be prepared using the ingredients mentioned above under tablets and capsules in a conventional manner using, e.g., a mixer, a fluid bed apparatus, or spray drying equipment.

Dosages

The dosage of any conjugate or composition described herein or identified using the methods described herein depends on several factors, including: the administration method, the disease (e.g., cancer) to be treated,

the severity of the disease, whether the cancer is to be treated or prevented, and the age, weight, and health of the subject to be treated.

With respect to the treatment methods of the invention, it is not intended that the administration of a vector, conjugate, or composition to a subject be limited to a particular mode of administration, dosage, or frequency of dosing; the invention contemplates all modes of administration. The conjugate, or composition may be administered to the subject in a single dose or in multiple doses. For example, a compound described herein or identified using screening methods of the invention may conjugate be administered once a week for, e.g., 2, 3, 4, 5, 6, 7, 8, 10, 15, 20, or more weeks. It is to be understood that, for any particular subject, specific dosage regimes should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the vector, conjugate, or composition. For example, the dosage of a conjugate can be increased if the lower dose does not provide sufficient activity in the treatment of a disease or condition described herein (e.g., cancer). Conversely, the dosage of the compound can be decreased if the disease (e.g., cancer) is reduced or eliminated.

While the attending physician ultimately will decide the appropriate amount and dosage regimen, a therapeutically effective amount of a vector, conjugate, or composition described herein, may be, for example, in the range of 0.0035 μg to 20 μg/kg body weight/day or 0.010 μg to 140 μg/kg body weight/week. Desirably a therapeutically effective amount is in the range of 0.025 μg to 10 μg/kg, for example, at least 0.025, 0.035, 0.05, 0.075, 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, 8.0, or 9.0 μg/kg body weight administered daily, every other day, or twice a week. In addition, a therapeutically effective amount may be in the range of 0.05 μg to 20 μg/kg, for example, at least 0.05, 0.7, 0.15, 0.2, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 10.0, 12.0, 14.0, 16.0, or 18.0 μg/kg body weight administered weekly, every other week, or once a month. Furthermore, a therapeutically effective amount of a

compound may be, for example, in the range of 100 $\mu g/m^2$ to 100,000 $\mu g/m^2$ administered every other day, once weekly, or every other week. In a desirable embodiment, the therapeutically effective amount is in the range of 1,000 $\mu g/m^2$ to 20,000 $\mu g/m^2$, for example, at least 1,000, 1,500, 4,000, or 14,000 $\mu g/m^2$ of the compound administered daily, every other day, twice weekly, weekly, or every other week.

The following examples are intended to illustrate, rather than limit the invention.

Example 1

Distribution and pharmacokinetics of conjugates

The effect of conjugation of an agent to a vector on distribution of the agent can be evaluated by administering a labeled polypeptide or conjugate to an animal and measuring distribution of the polypeptide or conjugate to organs. In one example, either ^{3}H-Taxol (5 mg/kg) or ^{125}I-Taxol-Angiopep-1 (TxlAn-1) (10 mg/kg, equivalent to 5 mg Taxol/kg) to mice. Similar experiments can be performed with any of Angiopep-3, Angiopep-4a, Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7. Here the unconjugated anticancer agent and the conjugates are injected intravenously to mice as a bolus. Tissues are collected at different times (0.25, 0.5, 1, and 4 hrs) and homogenized. To quantify the amount of ^{3}H-Taxol, tissue homogenates are digested with tissue solubilizer, and 10 ml of liquid scintiliator was added to samples. The amount of the ^{125}I labeled conjugate, in the different tissues is measured after TCA precipitation. Radioactivity associated with the tissues is quantified. The area under the curve (AUC 0-4) is estimated using the Prism software and is plotted for the different tissues. Using an Angiopep-1 conjugate, the AUC 0-4 values obtained for the conjugate are higher than that of Taxol in various tissues including the brain, kidney, liver, and the eyes (Fig. 10A) indicating a higher accumulation of the conjugate in these tissues compared to the unconjugated

agent. The accumulation of the conjugate is much higher than unconjugated agent in the lung (Fig. 10B).

Results of a similar experiment conducted with the Taxol-Angiopep-2 conjugate are summarized in Table 4 below. Although there is difference with results obtained for the TxlAn-1 conjugate, the conjugate of Table 4 also accumulates in the lungs, brain, and liver more efficiently than unconjugated Taxol.

Table 4

	AUC 0-4 (μg/g of tissue)		
Tissue	**TxlAn-2**	**Taxol**	**Ratio (TxlAn-2/Taxol)**
Plasma	170	2.2	77.3
Brain	0.32	0.07	4.6
Lung	3.4	1.1	3.0
Kidney	11.2	8.0	1.4
Heart	5.0	2.5	2.0
Liver	513	22	23
Eye	0.99	0.57	1.7
Urine	35.7	88	0.4

Treatments equivalent to 5 mg/kg of Taxol

The kinetics of Taxol and Taxol-Angiopep-1 accumulation in the lung were studied (Fig. 11). The amount of the conjugate measured in the lungs at different times is much higher than for the unconjugated agent. Accumulation of the conjugate in the lung is also much higher than its concentration in the serum (plasma) at various times (Fig. 12). These results indicate that biodistribution or pharmacokinetics of an anticancer agent such as Taxol can be altered by conjugation to a vector of the invention (e.g., Angiopep-1 or 2).

Example 2

Angiopep-7 is not efficiently transported to the brain

Angiopep-7 is not efficiently transported across the blood-brain barrier. Measuring accumulation of Angiopep-2 and Angiopep-7 in the brain of rats 30

minutes following IV injection, Angiopep-7 was present in the brain at lower levels than either Angiopep-1 or Angiopep-2 (Fig. 13).

We have also examined accumulation of Angiopep-2 and Angiopep-7 in the brain using fluorescence microscopy after a 10 minute in situ brain perfusion. Here, 2 mM of Angiopep-2 or Angiopep-7, each conjugated to the Cy5.5 fluorescent marker, were injected into the carotid artery. The brains of the animals were then evaluated by fluorescence microcopy. In each case, the capillaries were stained with Vessel Green (FITC-lectin), the nuclei of brain cells were stained with DAPI (blue), and the Angiopeps were visualized using the Cy5.5 label (Fig. 14). From these sections, Angiopep-2 is observed to be localized within the brain, whereas Angiopep-7 is observed to be localized within the capillaries. Lower brain accumulation of Angiopep-7 as compared to Angiopep-2 is also observed in vivo. Each of Angiopep-2 and Angiopep-7 were conjugated to Cy5.5 and administered to rats. After 30 minutes, accumulation in the brain was measured using the fluorescence of the Cy5.5 indicator. On this basis, we conclude that Angiopep-7 is not efficiently transported across the BBB, whereas Angiopep-2 is transported across the BBB efficiently.

Example 3

Angiopep-7 is efficiently transported to liver, kidney lungs, spleen, and muscle

Using rats administered Angiopep-2 or Angiopep-7 conjugated to Cy5.5 as described in Example 2, the levels of Angiopep-7 and Angiopep-2 were measured in organs such as liver, kidney, and lung. In these organs, similar levels of polypeptide were observed with Angiopep-7 as compared to Angiopep-2. In view of these results, we believe that Angiopep-7 may be especially useful for delivery of agent to the liver, kidney, and lungs in applications where delivery to the brain is not desired.

To measure Angiopep-7 concentrations in animals, imaging studies of Angiopep-7 conjugated to Cy5.5 have been performed. Based on in vivo imaging studies 30 minutes following injection, accumulation of the Cy5.5 signal was observed in the kidneys, the liver, and the lungs (Fig. 15A). Ex vivo organ analysis 24 hours after injection of Angiopep-7 was performed, and compared to injection using Angiopep-2. These studies also revealed significant accumulation of both Angiopep-2 and Angiopep-7 in the liver, lungs, and kidneys. However, Angiopep-7 showed significant less brain accumulation as compared to Angiopep-2 (Fig. 15B). Based on these results, we believe Angiopep-7 is useful as a vector for agents into peripheral organs such as the liver, kidney, lungs, spleen, or muscle in applications where delivery to the brain is not desired.

Example 4

Transport of IgG conjugates

Brain uptake of these vectors was measured using the *in situ* brain perfusion experimental model. Results (shown in Table 5 below) revealed that lysines at position 10 and 15 are important in these vectors ability to cross the BBB.

Table 5: Brain uptake of Angiopeps measured by *in situ* brain perfusion

Vector	Parenchyma (ml/100 g)	Transport relative to Angiopep-1 (250 nM), expressed as percent
Angiopep-1 (50 nM)	34.9	155.46
Angiopep-1 (250 nM)	22.45	100.00
Angiopep-1 (dimer) (250 nM)	27.03	120.40
Angiopep-2 (250 nM)	19.62	87.39
Angiopep-3 (250 nM)	17.08	76.08
Angiopep-4a (250 nM)	17.57	78.26
Angiopep-4b (250 nM)	12.05	53.67
Angiopep-5 (250 nM)	11.82	52.65
Angiopep-6 (250 nM)	8.64	38.49
Angiopep-7 (250 nM)	2.99	13.32

The Angiopep polypeptides were conjugated to IgG using the SATA cross-linker and their transport capacity was studied using *in situ* brain perfusion. As shown in Fig. 16, Angiopep-2 is the most highly distributed conjugate in brain parenchyma. It also appears that polypeptide dimers are efficiently transported. Angiopep-7 was not efficiently transported into the brain.

Example 5

Effect of conjugates on cell growth in vitro

To test for the ability of conjugates including a vector and an anticancer agent to kill cancer cells, in vitro can be performed. In one example, Taxol (unconjugated) is shown to block the proliferation of gliobiastoma cells (U-87) with IC50 value of around 10 nM (Fig. 17). The effect of Taxol conjugated when conjugated a vector described herein is evaluated and compared to unconjugated Taxol. As shown in Table 6A, the IC50 values obtained for the Taxol-Angiopep-2 (TxlAn2) conjugate were very similar to that of

unconjugated Taxol in many cancer cells. Endothelial cells from rat brain (RBE4) were less sensitive than the tested cancer cell lines. For comparison purposes, results obtained were expressed in term of Taxol concentration.

Most of theses cells (U-87, U-118, NC1-H460, A549) express LRP. This data is however unavailable for RBE4 cells. The anti-proliferation activity of the conjugate against cancer cells in vitro is assessed. In this assay, cancer cells (U87 and U118) are exposed for 48 hrs to an anticancer agent (e.g., Taxol) and conjugate (e.g., TxlAn2 (3:1) conjugate. Incorporation of [^{3}H]-thymidine in U87 and U118 cells decreases as a function of the concentration of the agent. The values required to inhibit cell proliferation by 50% (1C50) are expressed. Results obtained from the proliferation assays indicate that the IC50 values required for the inhibition of cancer cell proliferation are expressed in nM and demonstrate that TxlAn2 (3:1) conjugate is 3 times more potent than paclitaxel, and are in the same range when reported in paclitaxel equivalent (Table 6B). Similar experiments can be performed using any of the vectors or conjugates described herein.

The capacity of a conjugate (e.g., TxlAn2 (3:1)) to block the proliferation of other cancer cell types is also estimated. Such assays can be performed in lung cancer cells (NC1-H460) as well as the breast cancer cell line (MDA-MB231, MDA-MB-468, HCC-1954, BT-474) in hepatocarcinomas (SK-Hepl) and glioblastomas (U-87MG). We have shown that such cells are also very sensitive to TxlAn2 (3:1) conjugate (Table 6B).

Table 6A. Effect of conjugate on cell proliferation.

Cell lines	IC50 (nM)	
	Taxol	Taxol-Angiopep-2 (3:1)
Glioblastomas		
5 U-87	9.5	9.7
U-118	7.2	8.1
Lung carcinoma		
NCI-H460	9.3	12.5
A549	3.6	6.0
10 Calu-3	17.2	25.0
Endothelial cells		
RBE4	137	139

Table 6B: In vitro cytotoxicity of Taxol and TxlAn2 (3:1) conjugate

	Paclitaxel				TxlAn2 (3:1) conjugate			
Cell line	IC50 (nM)		Residual survival %		IC50 (nM)		Residual survival %	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
BT-474	62.86	-	52.24	3.87	40.22	-	51.83	1.70
HCC1954	6.12	-	48.82	11.17	8.26	3.37	44.08	8.63
MDA-MB-231	17.61	-	45.62	13.89	28.16	-	46.95	7.50
MDA-MB-468	13.52	-	54.26	10.34	1.41	-	55.34	5.55
NCI-H460	6.61	3.35	24.98	10.80	12.68	10.08	30.44	9.36
SK-HEP-1	8.84	-	55.18	13.17	7.83	-	54.09	11.03
U-87 MG	12.94	2.49	40.35	2.40	17.75	10.82	44.13	1.85

Example 6

Inhibition of tumor growth in vivo (U-87)

The ability of a conjugate to inhibit tumor growth can be evaluated in an in vivo model (see, e.g., Fig. 18A). U-87 cells are subcutaneously implanted in the right flank of mice and, on day 3 post-implantation, mice are injected with the vehicle (DMSO/Ringer:80/20; control), an unconjugated agent (e.g., Taxol (5 mg/kg)) or a the agent as part of a conjugate (e.g., Taxol-Angiopep-2 (10 mg/kg) equivalent to 5 mg Taxol/kg. Tumor growth inhibition is more pronounced in mice treated with the conjugate than in mice treated with the unconjugated anticancer agent.

In one example, at day 17 post-implantation, tumor growth was inhibited by more than 75% using the TxlAn2, whereas tumor growth was inhibited by 34% using the unconjugated agent (Table 7). Thus, the conjugates are more efficient than unconjugated agents at inhibiting tumor growth in vivo. Overall, a 2.2-fold tumor growth inhibition level was measured for the conjugate compared to the unconjugated agent.

Table 7. Inhibition of tumor growth with conjugates.

Groups	**Tumor volume (mm^3)** (mean ± sem) Days post-injection		**Tumor growth** Δ (mm^3)	**Inhibition (%)**	**T/C (%)**
	Day 0*	Day 14**			
Control	79 ± 7	289 ± 50	203 ± 47		100
Taxol (5 mg/kg)	74 ± 5	219 ± 52	134 ± 55	34	66
TxlAn2 (3:1) (10 mg/kg)	88 ± 9	144 ± 27	56 ± 32	73	27

Treatment equivalent to 5 mg/kg of Taxol
*corresponds to 3 days post-implantation (first treatment)
**corresponds to 17 days post-implantation (after 4 treatments)

Example 7

Inhibition of tumor growth in vivo (hepatocarcinoma)

In vivo studies are conducted to determine whether a conjugate can inhibit the growth of hepatocarcinoma cells (SK-Hep 1) implanted subcutaneously. Nude mice receive a subcutaneous injection of 2.5×10^6 human SK-Hep 1 cells in their right flank. Treatments are started when the size of the implanted tumor reached approximately 200 mm^3. Animals receive treatment with a conjugate or vehicle by intraperitoneal injections.

Results from an experiment performed with a TxlAn2 (3:1) administered at 80 mg/kg by intraperitoneal injection are shown in Fig. 18B; treatments are

indicated by black arrows. In this experiment, treatments were given twice a week for five treatments maximum at the dose indicated of 80 mg/kg. TxlAn2 (3:1) conjugate administered i.p. shows high efficacy in inhibiting the growth of hepatocarcinomas, a type of cancer usually not sensitive to Taxol® (Fig. 18B).

Example 8

Methods for evaluating conjugate activity

The following methods were used in Examples described herein.

Cell proliferation assay

For the in vitro cell proliferation assay, between 2.5 and 5 x 10^4 of U87 or A549 cells are seeded in a 24 well tissue culture microplate in a final volume of 1 ml of medium with 10% serum and incubated for 24 hours at 37 °C and 5% CO_2. The medium is then replaced with serum-free medium and incubated overnight. The next morning the agent is freshly dissolved in dimethyl sulfoxide (DMSO) and the medium is replaced with complete medium containing the agent at different concentrations in triplicates. The final concentration of DMSO is 0.1%. The control used is a microplate well with cells and without agent. The cells are incubated for 48 to 72 hrs at 37 °C and 5% CO_2. After the incubation, the medium is changed and replaced with 1 ml of complete medium containing [^{3}H]-thymidine (1 pCi/assay). The plate is incubated at 37 °C and 5% CO_2 for 4 hrs. The medium is removed, and the cells are washed with PBS at 37 °C. The cells are fixed with a mix of ethanol:acetic acid (3:1), are washed with water, and precipitated 3 times with 10% of ice-cold TCA (trichloroacetic acid). Finally 500 µl of PCA (perchloric acid) is added to the wells and the microplates are heated for 30 min at 65 °C and 30 min at 75 °C. The contents of each well is then transferred in a scintillation vial with 10 ml of scintillation cocktail and the activity is measured

in CPM (count per minute) on a liquid scintillation counter Tri-Carb from Packard.

Iodination of polypeptides

Polypeptides are iodinated with standard procedures using iodo-beads from Sigma. Briefly polypeptides are diluted in 0.1 M phosphate buffer, pH 6.5 (PB). Two iodo-beads are used for each protein. These beads are washed twice with 3 ml of PB on a Whatman filter and re-suspended in 60 µl of PB. ^{125}I (1 mCi) from Amersham-Pharmacia biotech was added to the bead suspension for 5 min at room temperature. Each iodination was initiated by the addition of the polypeptide (100 µg). After an incubation of 10 min at room temperature, the free iodine was removed by HPLC.

Subcutaneous implantation

In order to estimate the efficiency of the conjugates and formulations on tumor growth, we developed a subcutaneous model of glioblastomas. In this model, 2.5 x 10^6 cells in 100 µl of cell medium without serum containing 1% methylcellulose are subcutaneously injected in the mice flank. The tumor is clearly visible and can be measured using a vernier caliper. The estimated tumor volume was then plotted as a function of time.

In situ mouse brain perfusion

The uptake of [^{125}I]-polypeptides to the luminal side of mouse brain capillaries is measured using the in situ brain perfusion method adapted in our laboratory for the study of agent uptake in the mouse brain. Briefly, the right common carotid of ketamine/xylazine (140/8 mg/kg i.p.) anesthetized mice is exposed and ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid is then catheterized rostrally with polyethylene tubing filled with heparin (25 U/ml) and mounted on a 26-gauge needle. The syringe containing the perfusion fluid ([^{125}I] -

polypeptides or [^{14}C]-inulin in Krebs/bicarbonate buffer at a pH7.4 gassed with 95% O_2 and 5% CO_2) is placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus) and connected to the catheter. Prior to the perfusion, the contralateral blood flow contribution is eliminated by severing heart ventricles. The brain is perfused for the indicated times at a flow rate of 1.15 ml/min. After 14.5 min of perfusion, the brain is further perfused for 60 s with Krebs buffer to wash the excess of [^{125}I]-proteins. Mice are then decapitated to terminate perfusion and the right hemisphere is isolated on ice before being subjected to capillary depletion. Aliquots of homogenates, supernatants, pellets and perfusates are taken to measure their contents in [^{125}I]-conjugates by TCA precipitation and to evaluate the apparent volume of distribution.

Example 9

Mechanism of action of conjugates

Mechanism of action studies of conjugated therapeutic agents may also be performed. In one example, lung cancer cells (NCI-H460) are incubated for 24 hrs with either free Taxol (30 nM) or a Taxol conjugate (e.g., TxlAn2 10 nM; equivalent to 30 nM of Taxol; Fig. 19)). After cells are labeled for β-tubulin using a secondary antibody linked to FITC, pictures are taken in visible and fluorescence modes. In this example, both Taxol and the Taxol conjugate have similar effects on β-tubulin leading to its polymerization. As exemplified in Fig. 20, the addition of Taxol and the Taxol conjugate induce a blockade of NCI-H460 cell in G2/M phase. These results suggest that TxlAn conjugate has a similar mechanism of action on cancer cells than Taxol.

Example 10

Preparation of conjugates

Conjugation of an agent to a vector described herein is exemplified in Figs. 23A-23B. In this example, Taxol is first activated into an N-succinimide (2'-NHS-Txl) derivative. The amines found, for example, in a vector (e.g.,

amino-terminal amine or lysine residues) are reacted on the activated Taxol to form a peptide bond (amide bond). If multiple amine are available, this reaction produces multiple combinations of conjugates, e.g., with the addition of 1, 2 or 3 Taxols to the polypeptide, depending on the molar ratio used. The conjugation products are analyzed by HPLC, and conjugation is confirmed by Mass spectroscopy (Maldi-Tof). Taxol is found to be releasable from the vector by cleavage of the ester bond with an esterase.

Any vector described herein can be conjugated to an agent using this method. The production of the TxlAn2 (3:1) conjugate was carried out by directly adding 1 mole equivalent of Angiopep-2 to a solution of 2.5 moles equivalent of 2'-NHS-Taxol. The reaction was performed in 68% DMSO in Ringer solution (pH 7.3) for 1 hr at 12 °C. After removing the cold bath, the reaction was allowed to continue for about 22 hrs at room temperature (Fig. 24). Angiopep-2, 2'-NHS-Taxol and TxlAn2 (3:1) conjugate are shown on the chromatogram by arrows. Aliquots of the reaction were sampled and analyzed by HPLC after 25 min, 2 hrs 15 min, 5 hrs and 23 hrs as indicated in Fig. 24 The peaks of Angiopep-2, 2'-NHS-Taxol and TxlAn2 (3:1) conjugate are shown by arrows on the chromatogram. Results of Fig. 24 illustrate the disappearance of Angiopep-2 and 2'-NHS-Taxol during the reaction mainly to the profit of the TxlAn2 (3:1) conjugate.

This mixture of products was separated by hydrophobic chromatography on a RPC 300 mm column with a flow rate at 4 ml/min using AKTA-explorer (Fig. 25). For the peak that corresponds to the TxlAn2 (3:1) conjugate, fractions were pooled, analyzed by HPLC and MS. In Fig. 25, the upper chromatogram corresponds to the running reaction at t=23 hrs whereas the lower one corresponds to the TxlAn2 (3:1) conjugate which has been confirmed by mass spectrometry (MW 5107) after AKTA purification.

Example 11

Antibody conjugation and uptake

Antibodies can be conjugated to aprotinin as described in U.S. Patent Application Publication No. 2006/0189515. Similar results can be obtained with any vector described herein.

We therefore conjugated Angiopep-2 to IgG using SATA. Following conjugation, about 3 to 5 molecules of Angiopep-2 are associated with both the light and heavy chains of IgG (Fig. 26). Therefore, using this approach, 2 to 6 molecules of vector (e.g., Angiopep-1, Angiopep-2) are expected to be conjugated for each molecule of antibody (heavy and light chains).

Conjugation of Angiopep-2 using sulfo-EMCS was also performed, as illustrated in Fig. 4. The brain distribution of IgG-Angiopep-2 conjugates coupled via sulfhydryl groups using EMCS-Angiopep-2 were tested in various brain tissues (total brain, capillaries, parenchyma). There is higher (about three fold difference) brain uptake of [^{125}I]-IgG-Angiopep-2 conjugate than that of unconjugated [^{125}I]-IgG (see Fig. 27). The conjugation of IgG with Angiopep-2 therefore increases IgG accumulation in the brain *in vivo*.

The transport of Angiopep-2-IgG conjugates coupled using the SATA method was also tested using *in situ* brain perfusion experiments. IgG-An2 conjugates are transported into brain tissues (Fig. 28). IgG-Angiopep-2 conjugates accumulated in the parenchyma about 50 times greater than IgG.

Following conjugation of IgG with vectors, SDS-polyacrylamide gel electrophoresis, immunodetection, and autoradiography were performed to ensure proper conjugation; see, e.g., Fig. 29, which shows only [^{125}I]-IgG-Angiopep conjugates were detected. There was no evidence of free Angiopep-2 conjugate, showing the efficiency of the conjugation approach.

Example 12

Coupling of an anti-EGFR antibody to a vector

Signaling via the epidermal growth factor receptor induces cell proliferative signals and is associated with the transformation of normal to malignant cells. Several mutations in EGFR may be detected in tumor cells. One of the most common mutation of EGFR is the EGFRvIII mutation wherein amino acids 6-273 are deleted.

To show that coupling of vectors of the invention to antibodies other than IgG was possible, and that coupling maintained the antibody function, an EGFR antibody (monoclonal 528 available from ATCC) was cross-linked to the vector using the cross-linker SATA as an exemplary therapeutic polypeptide. The biological activity of the coupled EGFR antibody was tested by staining EGFR positive U87 cells. Similar detection of EGFR in U87 using both uncoupled and coupled EGFR antibodies by FACS analysis was demonstrated (Fig. 30) showing that coupling of the antibodies of the vectors of the invention did not alter their activity.

In Table 8, we show that the antibodies are not inactivated by conjugation and present the same affinity for binding in an in vitro assay.

Table 8: Kinetics analysis of the conjugate binding to EGFR receptor by FACS

	Anti-EGFR	Anti-EGFR-Angiopep-2
Bmax (RFU)	23.6	22.0
Half saturation (nM)	1.7	1.8

Transport of EGFR antibody conjugates (e.g., anti-EGFR-Angiopep-2) across the BBB was then measured using a labeled conjugate. The uptake of anti-[^{125}I]-EGFR-Angiopep-2 to the luminal side of mouse brain capillaries was measured using the *in situ* brain perfusion method as shown in Fig. 31. There was higher brain uptake for [^{125}I]-EGFR-Angiopep-2 conjugate than that of unconjugated [^{125}I]-EGFR Ab in all tissues tested. Therefore, the conjugation of EGFR Ab with Angiopep-2 increases its accumulation in the brain

parenchyma *in vivo*. These conjugates represent an interesting avenue as several different tumor types were show to express EGFR at various levels (see Table 9).

Table 9

Tumor type	Tumors with expressed EGFR (%)
Head and Neck	90–95
Breast	82–90
Renal carcinoma	76–89
Cervix/uterus	90
Esophagael	43–89
Pancreatic	30–89
Non-small-cell lung	40–80
Prostate	40–80
Colon	25–77
Ovarian	35–70
Glioma	40–63
Bladder	31–48
Gastric	4–33

Example 13

Coupling and transport of an anti-VEGF antibody to a vector

To further demonstrate the versatility and applicability of antibody coupling to vectors of the invention, another exemplary therapeutic antibody, Avastin, was used. Avastin is an anti-VEGF recombinant humanized monoclonal IgG1 kappa isotype antibody available from Roche Biochemical which binds to and inhibits biologically active forms of vascular endothelial growth factor (VEGF).

Following conjugation, the transport of Avastin conjugates across the BBB was then measured using the in situ brain perfusion method. There is higher brain uptake for [^{125}I]-Avastin-Angiopep-2 conjugate than that of unconjugated [^{125}I]-Avastin (Fig. 32).

Conjugation of therapeutic antibodies (such as Avastin or MAb 528 available from ATCC) to vectors (e.g., Angiopep-1, -2, -3, -4a, -4b, 5, and 6) is therefore a useful strategy for their transport into the brain.

Example 14

Dimer transport

Vector dimers can also be transported. In one example, Angiopep-1 was incubated at 4 °C for at least 12 hours, thereby causing multimerization/dimerization of the polypeptide. The transcytosis capacity of the Angiopep-1 dimer was evaluated *in vitro*. Angiopep-1 dimer is transcytosed better than IgG. Moreover, as shown in Table 5, the dimeric form of Angiopep-1 shows a distribution volume higher than that of Angiopep-1. Thus, vector dimers can be transported across the BBB.

Example 15

Conjugation of an antibody to more than one vector

Typically, cross-linking reactions yield between 1 to 6 molecules of vector per one molecule of antibody, heavy and light chains.

The level of conjugation of Angiopep-2 to IgGs was estimated using an assay allowing titration of the amount of sulfhydryl groups after deprotection of SATA on the amine of the IgGs before and after reaction with the maleimide group on the N terminal of Angiopep-2. By changing the relative concentration of the different reactants used in the conjugation protocol the amount of Angiopep-2 conjugated to IgGs may be optimized.

Using *in vivo* brain perfusion experiments, the higher the level of conjugation, the higher was the parenchymal uptake of conjugates (Fig. 33). Therefore, it may be desirably to have a larger number of vector per molecule. As such, more than 6 vectors may be used for transporting a compound (agent) across the BBB.

Other embodiments

The content of each publication, patent, and patent application mentioned in the present application is incorporated by reference, including U.S. Application Nos. 11/807,597, filed May 29, 2007, and 11/807,917, filed

May 30, 2007, and U.S. Provisional Application No. 61/008,825, filed December 20, 2007.

Although the invention has been described in details herein and illustrated in the accompanying drawings, it is to be understood that the invention is not limited to the embodiments described herein and that various changes and modifications may be effected without departing from the scope or spirit of the invention.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

What is claimed is:

CLAIMS

1. A polypeptide comprising an amino acid sequence having the following formula:

X1-X2-X3-X4-X5-X6-X7-X8-X9-X10-X11-X12-X13-X14-X15-X16-X17-X18-X19

wherein X1-X19 are any amino acid or are absent;
at least one of X1, X10, and X15 is Arg; and
said amino acid sequence has at least 50% identity to an amino acid sequence selected from the group consisting of Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7 (SEQ ID NOS:109-112).

2. The polypeptide of claim 1 comprising an amino acid sequence having at least 50% identity to the sequence of Angiopep-7.

3. The polypeptide of claim 1, wherein said identity is at least 70%.

4. The polypeptide of claim 1, wherein said identity is at least 90%.

5. The polypeptide of claim 1, wherein X10 is Lys or Arg, X15 is Lys or Arg, or both.

6. The polypeptide of claim 1, wherein X1 is Arg.

7. The polypeptide of claim 1, wherein X10 is Arg.

8. The polypeptide of claim 1, wherein X15 is Arg.

9. The polypeptide of claim 1, wherein X10 and X15 are Arg.

10. The polypeptide of claim 1, wherein X7 is Ser or Cys.

11. The polypeptide of claim 1, wherein X7 is Ser.

12. The polypeptide of claim 1 comprising an amino acid sequence selected from the group consisting of Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7.

13. The polypeptide of claim 12 comprising the amino acid sequence of Angiopep-7.

14. The polypeptide of claim 12 consisting of the amino acid sequence of selected from the group consisting of Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7.

15. The polypeptide of claim 14 consisting of the amino acid sequence of Angiopep-7.

16. The polypeptide of claim 1, wherein said polypeptide is transported to at least one cell or tissue selected from the group consisting of liver, lung, kidney, spleen, and muscle.

17. The polypeptide of claim 1, wherein said polypeptide is not efficiently transported across the blood-brain barrier (BBB).

18. The polypeptide of claim 17 comprising a sequence with at least 80% identity to Angiopep-7.

19. A composition comprising the polypeptide of claim 1.

20. The composition of claim 19 comprising a pharmaceutically acceptable carrier.

21. A conjugate comprising:

(a) a vector comprising a polypeptide of claim 1; and

(b) a therapeutic agent, diagnostic agent, or detectable label, wherein said agent or label is conjugated to said vector.

22. The conjugate of claim 21, wherein said vector comprises an amino acid sequence having at least 50% identity to the sequence of Angiopep-7.

23. The conjugate of claim 21, wherein said identity is at least 70%.

24. The conjugate of claim 21, wherein said identity is at least 90%.

25. The conjugate of claim 21, wherein X10 is Lys or Arg; X15 is Lys or Arg; or both.

26. The conjugate of claim 21, wherein X1 is Arg.

27. The conjugate of claim 21, wherein X10 is Arg.

28. The conjugate of claim 21, wherein X15 is Arg.

29. The conjugate of claim 21, wherein X10 and X15 are Arg.

30. The conjugate of claim 21, wherein X7 is Ser or Cys.

31. The conjugate of claim 21, wherein X7 is Ser.

32. The conjugate of claim 21, wherein said vector comprises an amino acid sequence selected from the group consisting of Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7.

33. The conjugate of claim 21, wherein said vector comprises the amino acid sequence of Angiopep-7.

34. The conjugate of claim 21, wherein said vector consists of an amino acid sequence selected from the group consisting of Angiopep-4b, Angiopep-5, Angiopep-6, and Angiopep-7.

35. The conjugate of claim 21, wherein said vector consists of the amino acid sequence of Angiopep-7.

36. The conjugate of claim 21, wherein said vector is conjugated to a therapeutic agent.

37. The conjugate of claim 21, wherein said therapeutic agent is an anticancer agent.

38. The conjugate of claim 21, wherein said vector is efficiently transported to at least one cell or tissue selected from the group consisting of liver, lung, kidney, spleen, and muscle.

39. The conjugate of claim 21, wherein said vector is not efficiently transported across the BBB.

40. The conjugate of claim 37, wherein said anticancer agent is selected from the group consisting of abarelix, aldesleukin, alemtuzumab, alitretinoin, allopurinol, altretamine, amifostine, anakinra, anastrozole, arsenic trioxide, asparaginase, azacitidine, BCG Live, bevacuzimab, bexarotene, bleomycin, bleomycin, bortezombi, bortezomib, busulfan, busulfan, calusterone, capecitabine, carboplatin, carmustine, celecoxib, cetuximab, chlorambucil, cisplatin, cladribine, clofarabine, cyclophosphamide, cytarabine, dacarbazine,

dactinomycin, actinomycin D, dalteparin, darbepoetin alfa, dasatinib, daunorubicin, daunomycin, decitabine, denileukin, Denileukin diftitox, dexrazoxane, docetaxel, doxorubicin, dromostanolone propionate, eculizumab, epirubicin, epoetin alfa, erlotinib, estramustine, etoposide (e.g., phosphate), exemestane, fentanyl, filgrastim, floxuridine, fludarabine, fluorouracil, 5-FU, fulvestrant, gefitinib, gemcitabine, gemtuzumab ozogamicin, goserelin, histrelin, hydroxyurea, ibritumomab tiuxetan, idarubicin, ifosfamide, imatinib, Interferon alfa-2b, irinotecan, lapatinib ditosylate, lenalidomide, letrozole, leucovorin, leuprolide, levamisole, lomustine, CCNU, meclorethamine (nitrogen mustard), megestrol, melphalan (L-PAM), mercaptopurine (6-MP), mesna, methotrexate, methoxsalen, mitomycin C, mitotane, mitoxantrone, nandrolone phenpropionate, nelarabine, nofetumomab, oprelvekin, oxaliplatin, paclitaxel, palifermin, pamidronate, panitumumab, pegademase, pegaspargase, pegfilgrastim, peginterferon alfa-2b, pemetrexed, pentostatin, pipobroman, plicamycin (mithramycin), porfimer, procarbazine, quinacrine, rasburicase, rituximab, sargramostim, sorafenib, streptozocin, sunitinib, sunitinib maleate, talc, tamoxifen, temozolomide, teniposide (VM-26), testolactone, thalidomide, thioguanine (6-TG), thiotepa, thiotepa, thiotepa, topotecan, toremifene, Tositumomab/I-131 (tositumomab), trastuzumab, trastuzumab, tretinoin (ATRA), uracil mustard, valrubicin, vinblastine, vincristine, vinorelbine, vorinostat, zoledronate, and zoledronic acid.

41. The conjugate of claim 40, wherein said agent is paclitaxel.

42. The conjugate of claim 37, wherein said agent is an antibody.

43. The conjugate of claim 42, wherein said antibody is selected from the group consisting of ABX-EGF (Panitimumab), OvaRex (Oregovemab), Theragyn (pemtumomabytrrium-90), Therex, Bivatuzumab, Panorex (Edrecolomab), ReoPro (Abciximab), Bexxar (Tositumomab), MAb, idiotypic

105AD7, Anti-EpCAM (Catumaxomab), MAb lung cancer (from Cytoclonal), Herceptin (Trastuzumab), Rituxan (Rituximab), Avastin (Bevacizumab), AMD Fab (Ranibizumab), E-26 (2^{nd} gen. IgE) (Omalizumab), Zevalin (Rituxan + yttrium-90) (Ibritumomab tiuxetan), Cetuximab, BEC2 (Mitumomab), IMC-1C11, nuC242-DM1, LymphoCide (Epratuzumab), LymphoCide Y-90, CEA-Cide (Labetuzumab), CEA-Cide Y-90, HumaRAD-HN (+ yttrium-90), MDX-101 (CTLA-4), MDX-210, MDX-210/MAK, Vitaxin, MAb 425, IS-IL-2, Campath (alemtuzumab), CD20 streptavidin, Avidicin, (albumin + NRLU13), Oncolym (+ iodine-131) Cotara (+ iodine-131), C215 (+ staphylococcal enterotoxin, MAb lung/kidney cancer (from Pharmacia Corp.), nacolomab tafenatox (C242 staphylococcal enterotoxin), Nuvion (Visilizumab), SMART M195, SMART 1D10, CEAVac, TriGem, TriAb, NovoMAb-G2 radiolabeled, Monopharm C, GlioMAb-H (+ gelonin toxin), Rituxan (Rituximab), and ING-1.

44. The conjugate of claim 21, wherein said conjugate accumulates more rapidly, accumulates to a higher concentration, exhibits reduced P-gp mediated efflux in a liver, lung, kidney, or spleen cell, as compared to said agent or label when not conjugated to said vector.

45. A composition comprising the conjugate of claim 21 and a pharmaceutically acceptable carrier.

46. A method of treating a subject having a cancer, said method comprising providing to said subject a conjugate of claim 37, in an amount sufficient to treat said subject.

47. The method of claim 46, wherein said cancer is a lung, liver, kidney, or spleen cancer.

48. The method claim 46, wherein said cancer is selected from the group consisting of hepatocellular carcinoma, breast cancer, cancers of the head and neck, lymphoma, mantle cell lymphoma, non-Hodgkins lymphoma, adenoma, squamous cell carcinoma, laryngeal carcinoma, cancers of the retina, cancers of the esophagus, multiple myeloma, ovarian cancer, uterine cancer, melanoma, colorectal cancer, bladder cancer, prostate cancer, lung cancer, non-small cell lung carcinoma, pancreatic cancer, cervical cancer, head and neck cancer, skin cancers, nasopharyngeal carcinoma, liposarcoma, epithelial carcinoma, renal cell carcinoma, gallbladder adenocarcinoma, parotid adenocarcinoma, endometrial sarcoma, and multidrug resistant cancers.

49. The method of claim 46, wherein said conjugate comprises a vector that is efficiently transported to at least one of the cells or tissues selected from the group consisting of liver, lung, kidney, spleen, or muscle.

50. The method of claim 46, wherein said conjugate comprises a vector that is not efficiently transported across the BBB.

51. The method of claim 50, wherein said conjugate comprises Angiopep-7.

52. The method of claim 46, wherein said anticancer agent is paclitaxel.

53. The method of claim 46, wherein said anticancer agent has increased efficacy or reduced side effects when conjugated to said polypeptide, as compared to said agent when not conjugated to said polypeptide.

54. The method of claim 46, wherein said subject is a human.

Peptide	Amino acid sequence	Charge
Angiopep	TFFYGGCRGKRNNFKTEEY	+2
# 67	TFFYGGCRGKRNNFKTEEY-amide	+2
# 76	TFFYGGCRGKRNNFKTKEY-amide	+3
# 91	RFKYGGCLGNKNNYLRLKY-amide	+5
# 5	TFFYGGCRAKRNNFKRAKY-amide	+6

Charge + : lysine (K), arginine (R)
Charge - : glutamic acid (E), aspartic acid (D)

Fig. 1

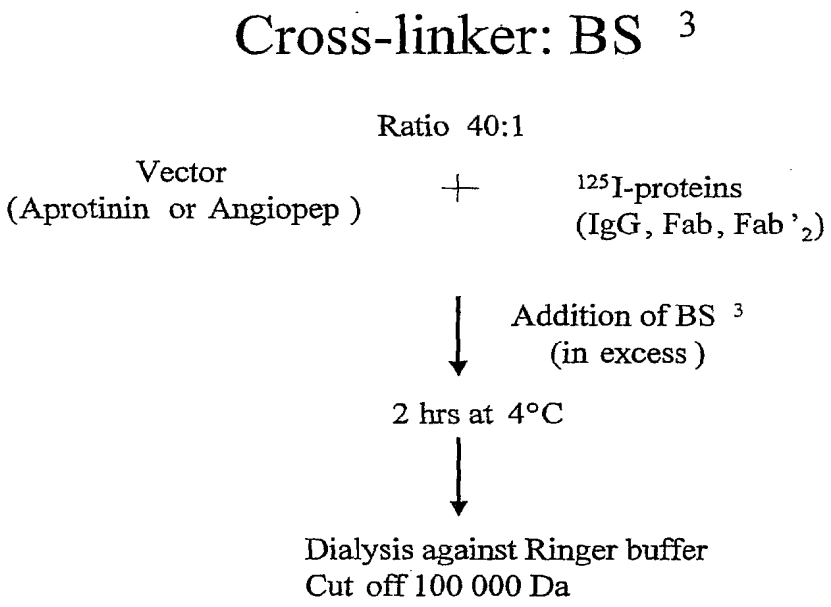

Cross-linker: BS 3
Ratio 40:1
Vector
(Aprotinin or Angiopep)
+
125I-proteins
(IgG, Fab, Fab '2)
Addition of BS 3
(in excess)
2 hrs at 4°C
Dialysis against Ringer buffer
Cut off 100 000 Da

Fig. 2

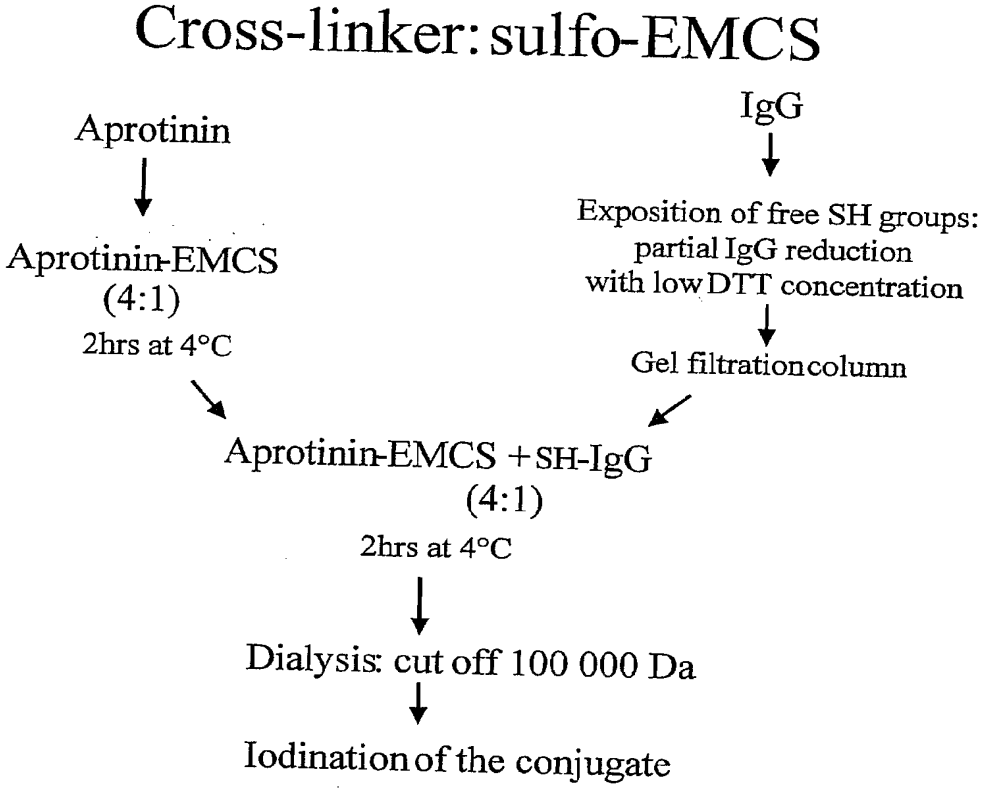

Cross-linker: sulfo-EMCS
Aprotinin
Aprotinin-EMCS
(4:1)
2hrs at 4°C
IgG
Exposition of free SH groups:
partial IgG reduction
with low DTT concentration
Gel filtration column
Aprotinin-EMCS + SH-IgG
(4:1)
2hrs at 4°C
Dialysis: cut off 100 000 Da
Iodination of the conjugate

Fig. 3

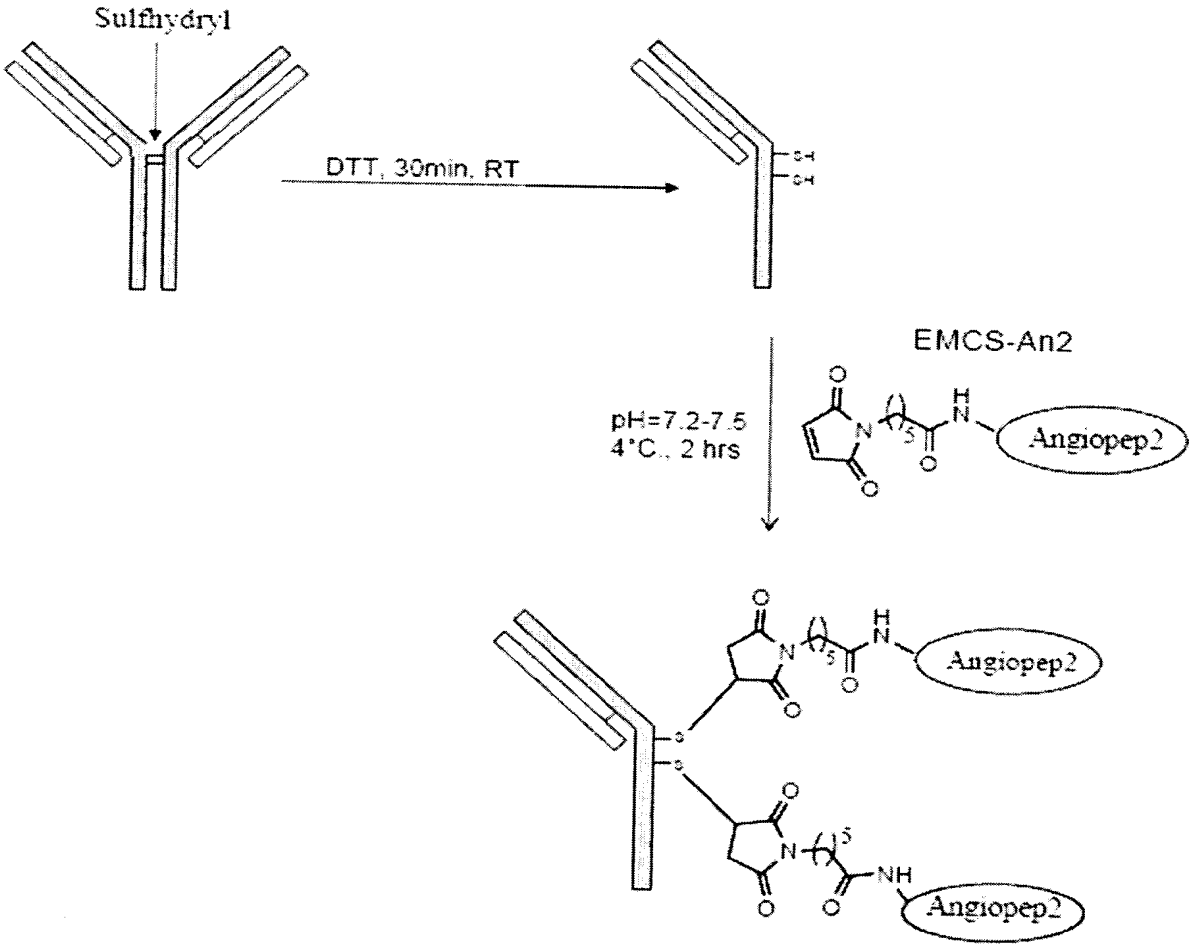

Sulfhydryl
DTT, 30min, RT
EMCS-An2
pH=7.2-7.5
4°C., 2 hrs
Angiopep2
Angiopep2
Angiopep2

Fig. 4

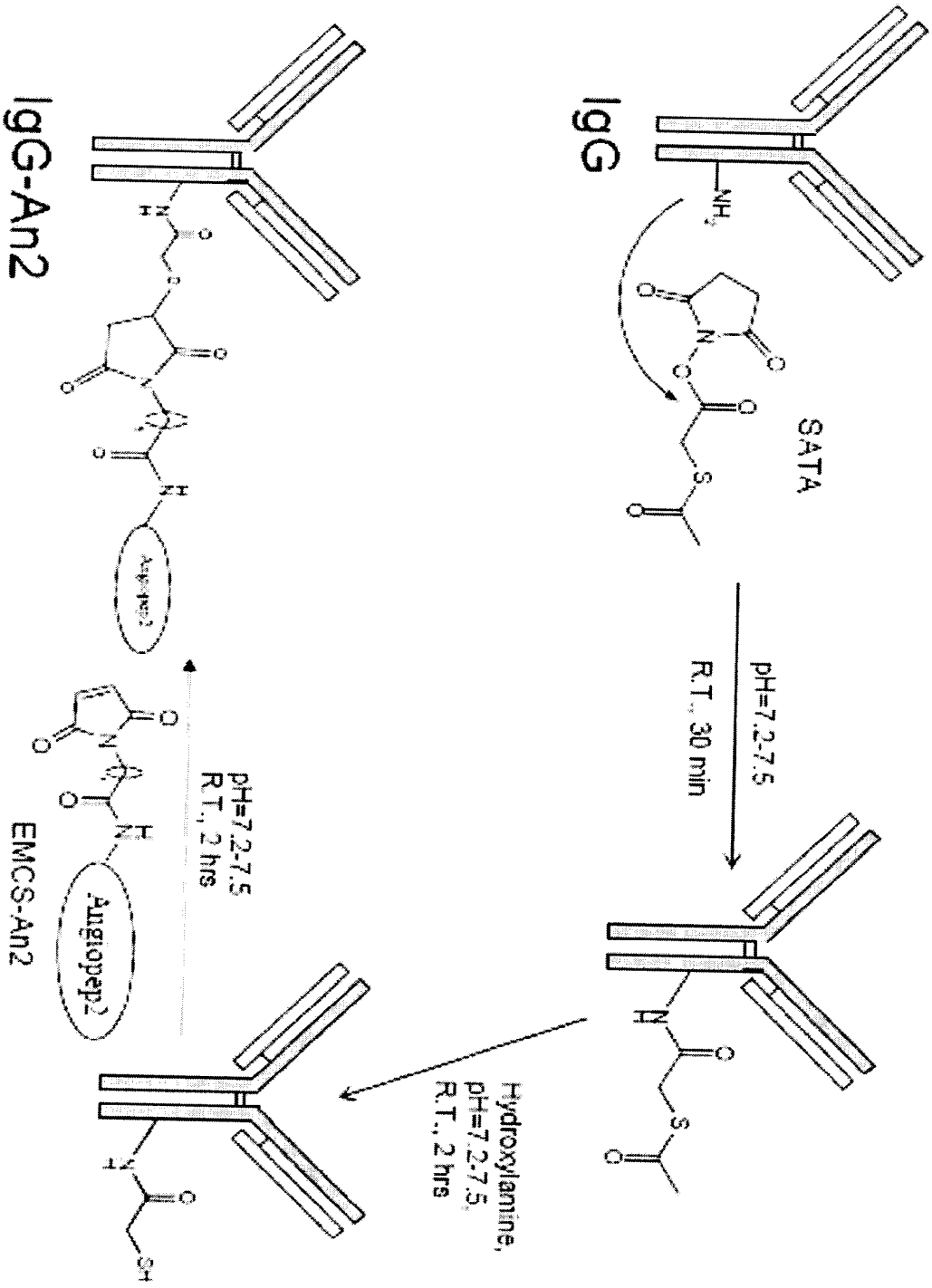

IgG
SATA
pH=7.2-7.5
R.T., 30 min
Hydroxylamine,
pH=7.2-7.5
R.T., 2 hrs
pH=7.2-7.5
R.T., 2 hrs
EMCS-An2
Angiopep2
IgG-An2

Fig. 5

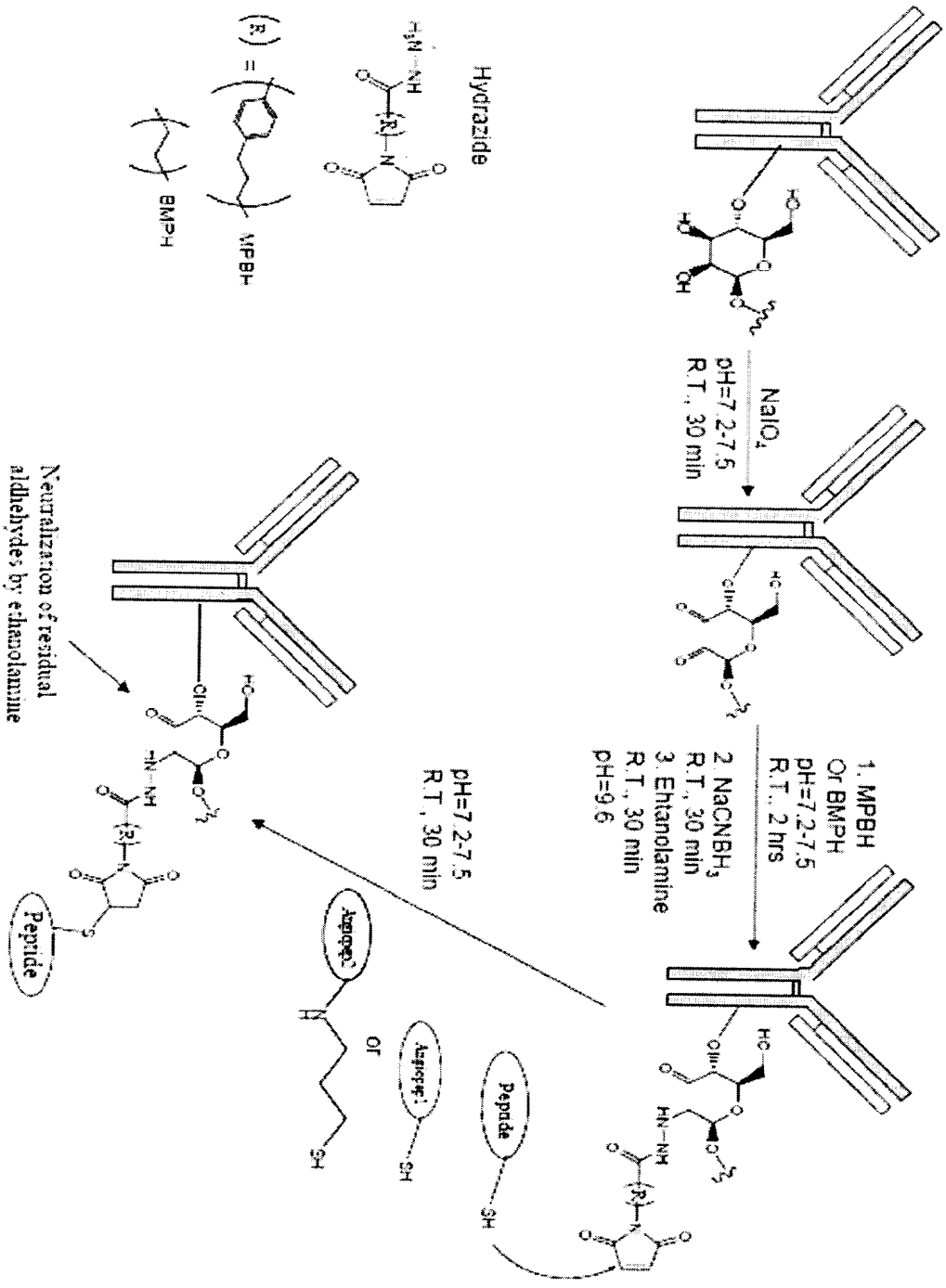
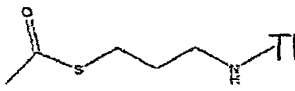

Hydrazide
BMPH
MPBH
NaIO4
pH=7.2-7.5
R.T., 30 min
1. MPBH
Or BMPH
pH=7.2-7.5
R.T., 2 hrs
2 NaCNBH3
R.T., 30 min
3 Ehtanolamine
R.T., 30 min
pH=9.6
pH=7.2-7.5
R.T., 30 min
Peptide
or
Neutralization of residual
aldhehydes by ethanolamine
Peptide

Fig. 6

1) 

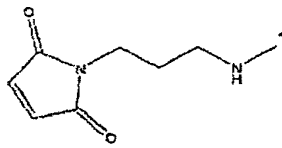
2) 

Fig. 7

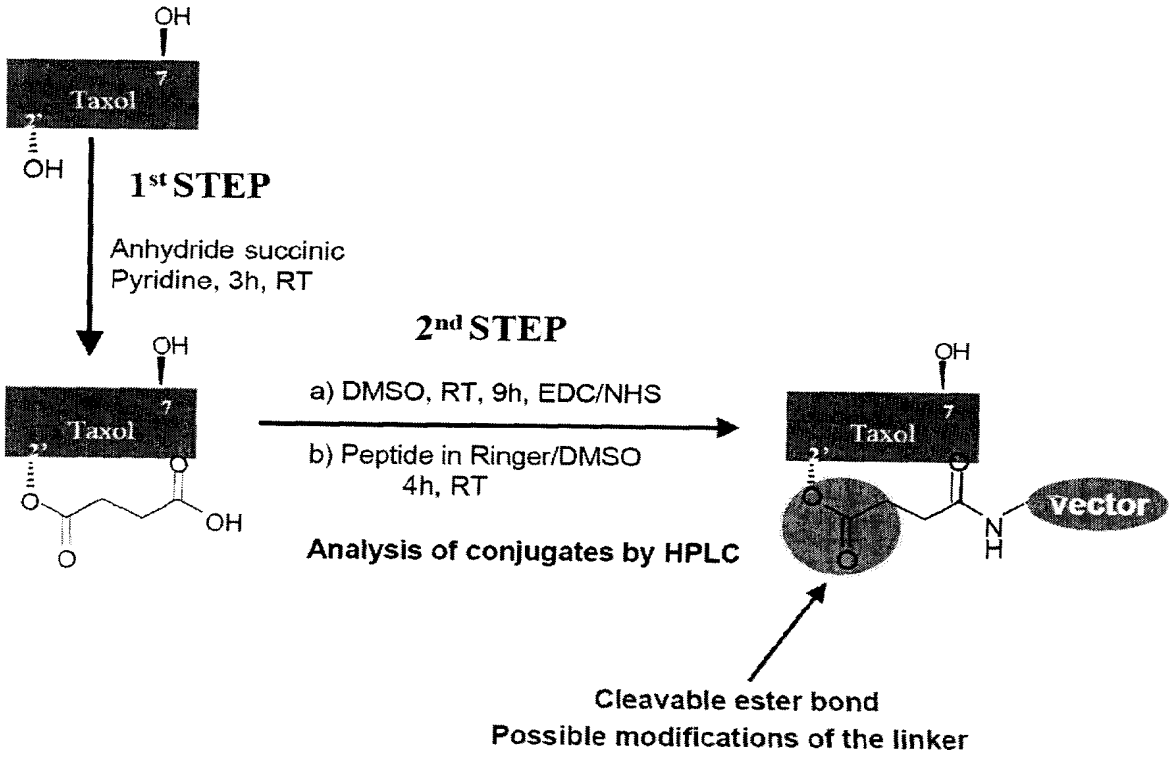

OH
Taxol
7
2'
OH
1st STEP
Anhydride succinic
Pyridine, 3h, RT
2nd STEP
a) DMSO, RT, 9h, EDC/NHS
b) Peptide in Ringer/DMSO
4h, RT
Analysis of conjugates by HPLC
Vector
Cleavable ester bond
Possible modifications of the linker

Fig. 8

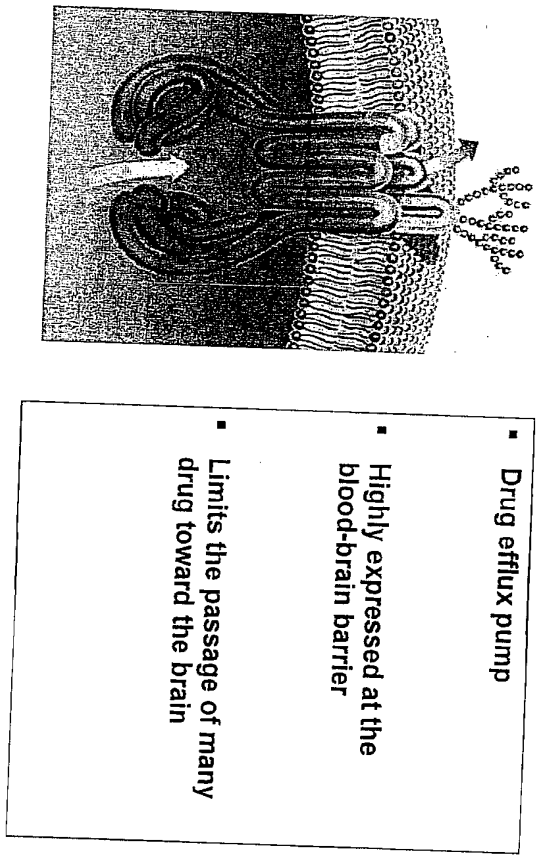

Drug efflux pump
Highly expressed at the blood-brain barrier
Limits the passage of many drug toward the brain

Fig. 9

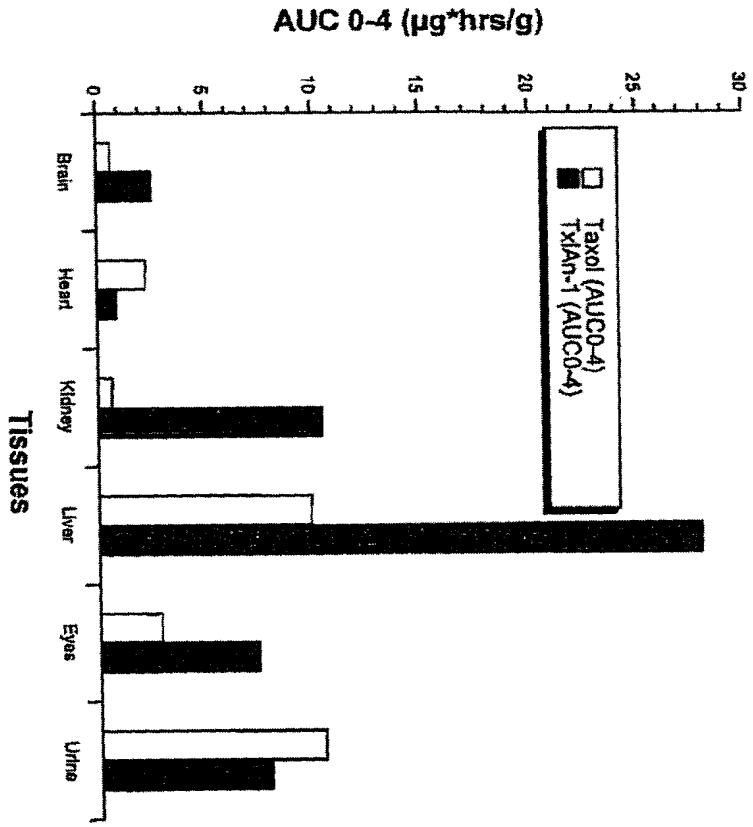


Fig. 10A

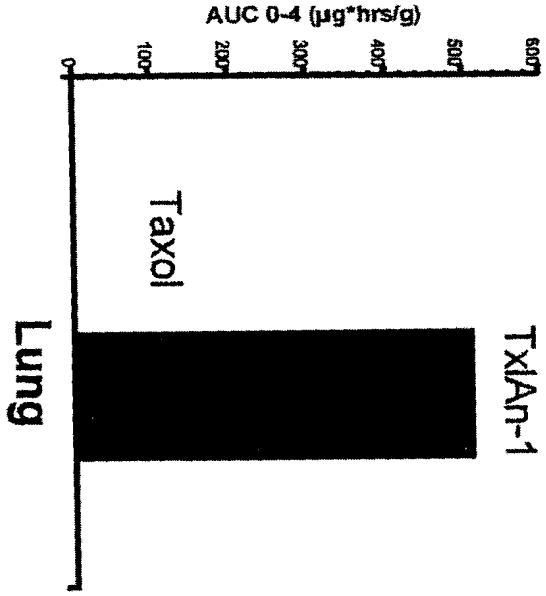


Fig. 10B

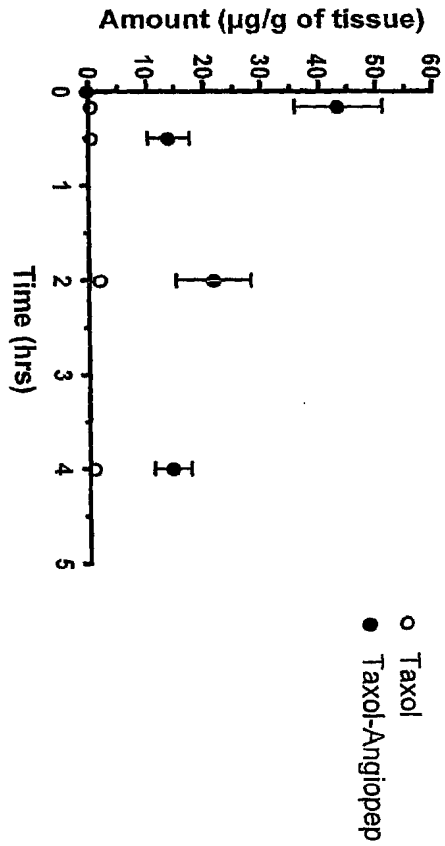

Amount (µg/g of tissue)
0
10
20
30
40
50
60
Time (hrs)
0
1
2
3
4
5
o Taxol
● Taxol-Angiopep

Fig. 11

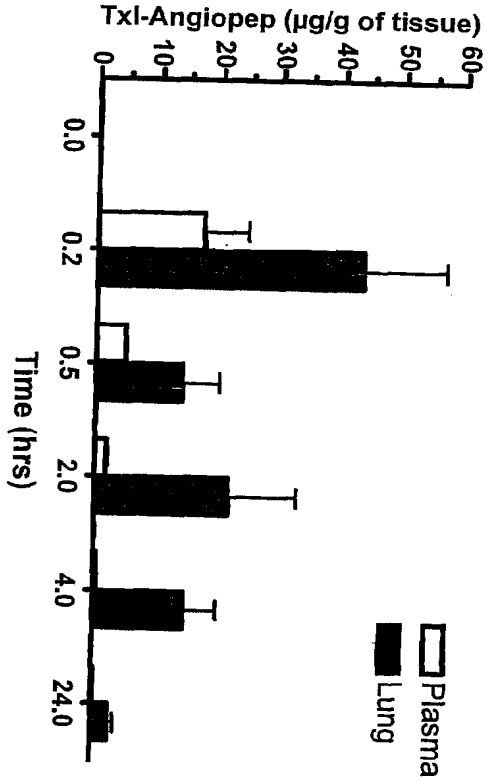

Txl-Angiopep (µg/g of tissue)
0
10
20
30
40
50
60
Time (hrs)
0.0
0.2
0.5
2.0
4.0
24.0
Lung
Plasma

Fig. 12

AngioPep-2

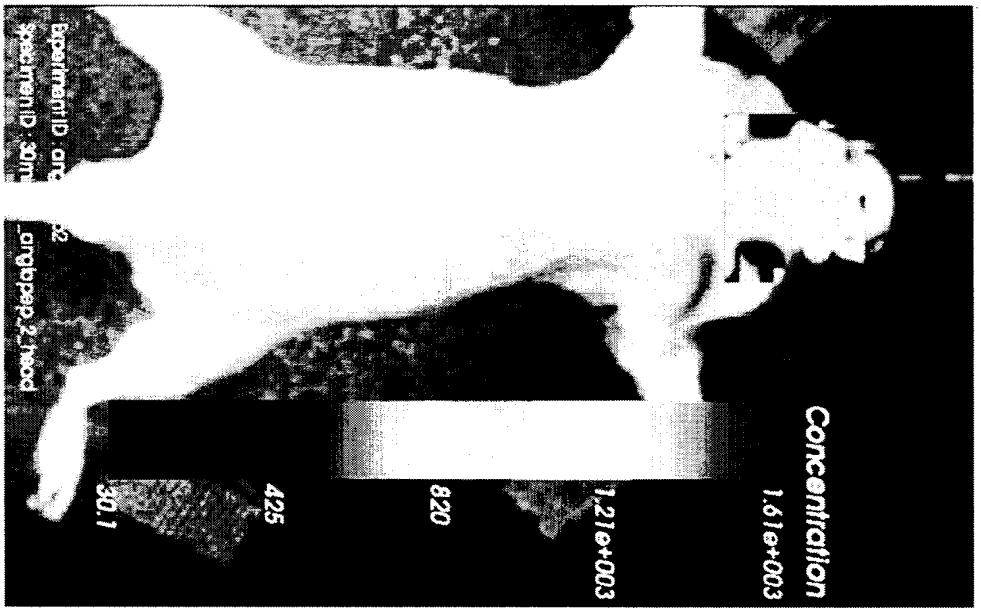


Fig. 13A

AngioPep-7

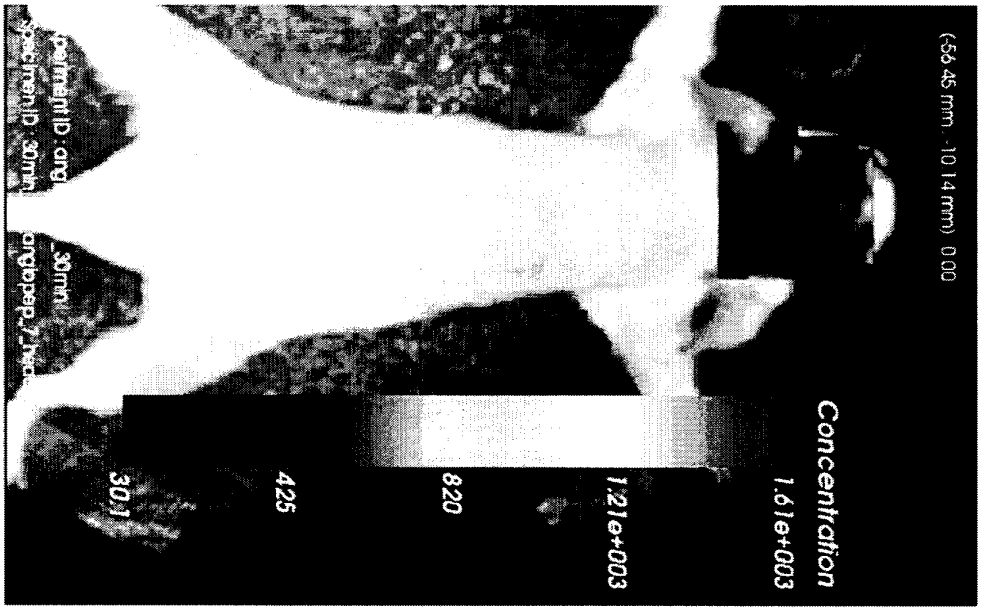


Fig. 13B

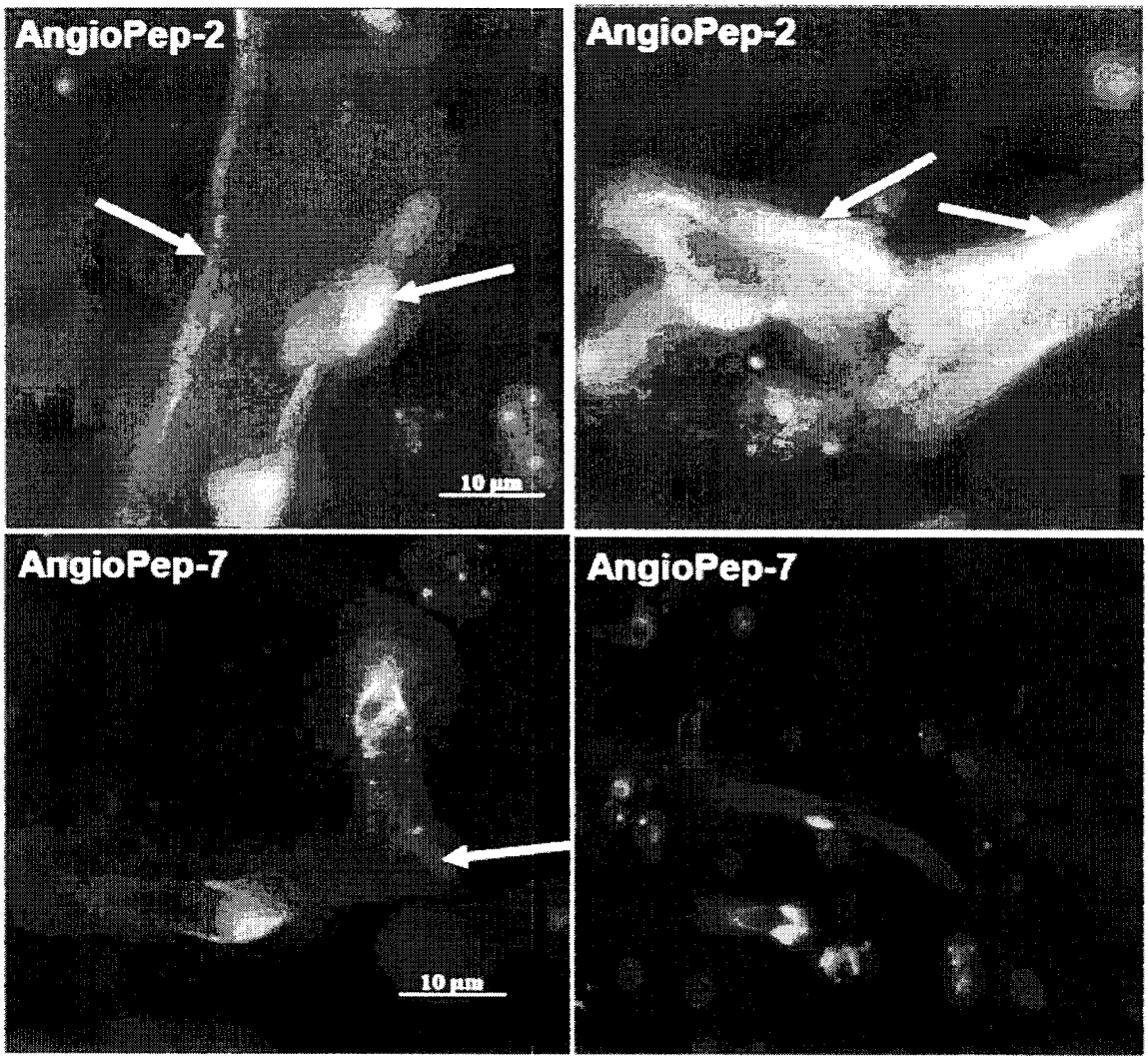


Fig. 14

Intensity

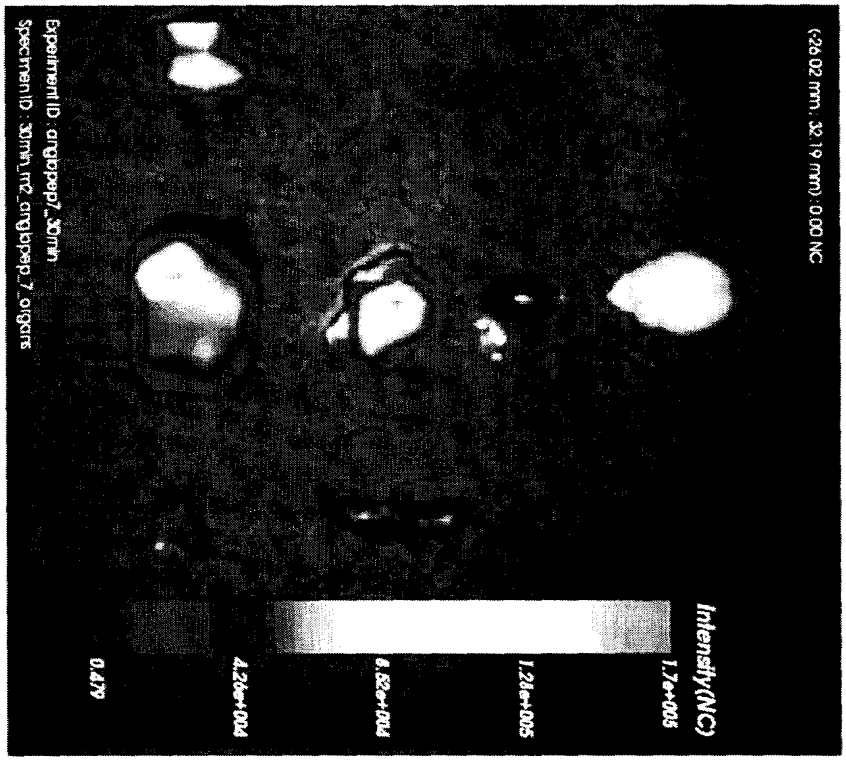


Fig. 15A

Lifetime

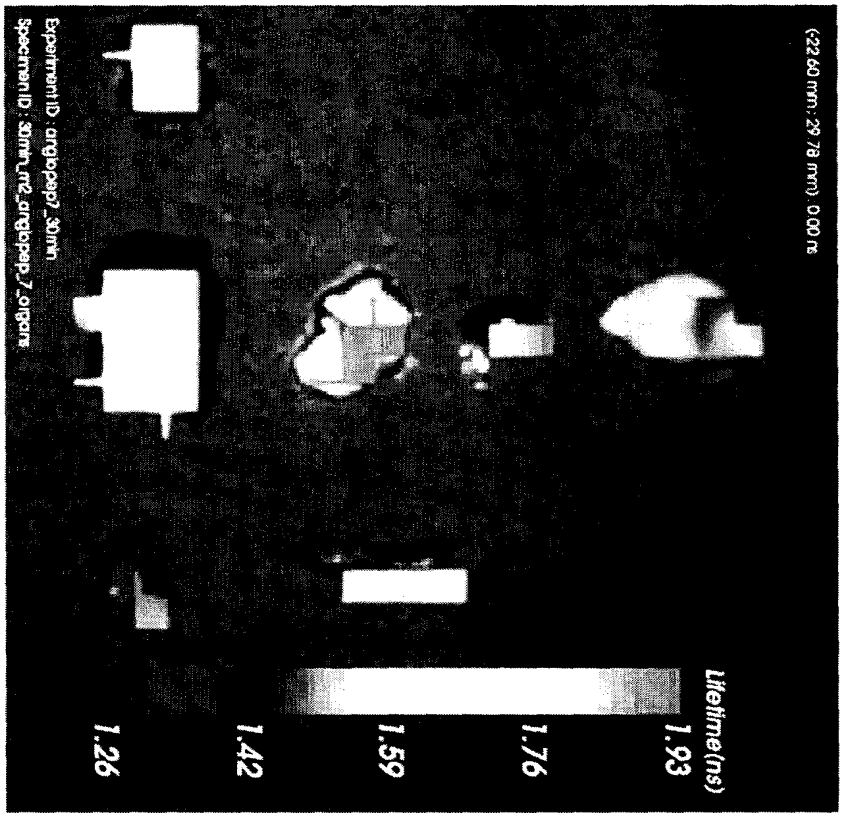


Fig. 15B

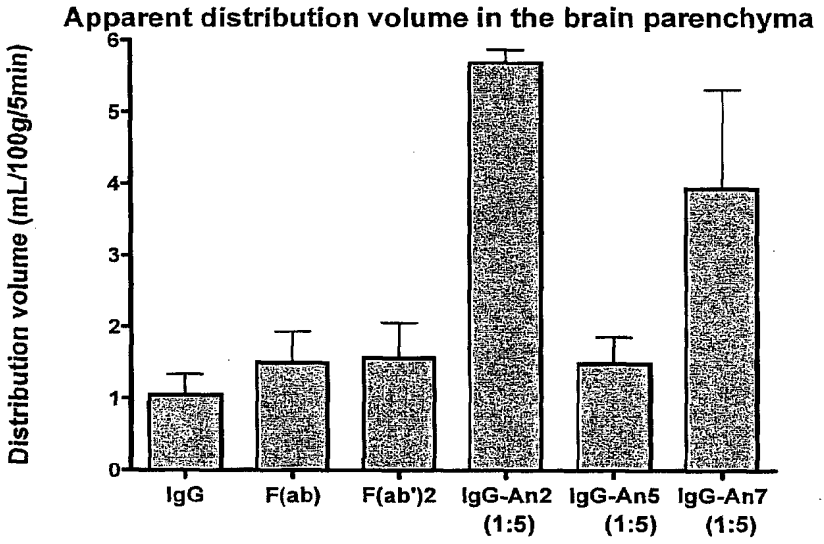

Apparent distribution volume in the brain parenchyma
Distribution volume (mL/100g/5min)
0
1
2
3
4
5
6
IgG
F(ab)
F(ab')2
IgG-An2 (1:5)
IgG-An5 (1:5)
IgG-An7 (1:5)

Fig. 16

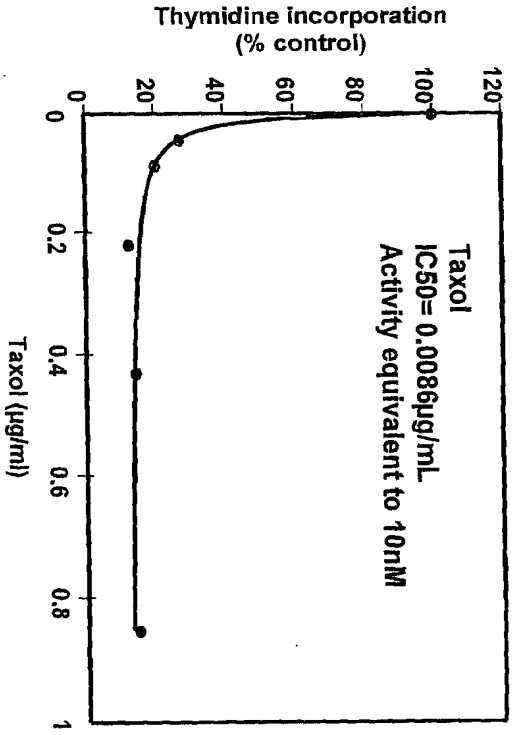

Thymidine incorporation
(% control)
0
20
40
60
80
100
120
Taxol (µg/ml)
0
0.2
0.4
0.6
0.8
1
Taxol
IC50= 0.0086µg/mL
Activity equivalent to 10nM

Fig. 17

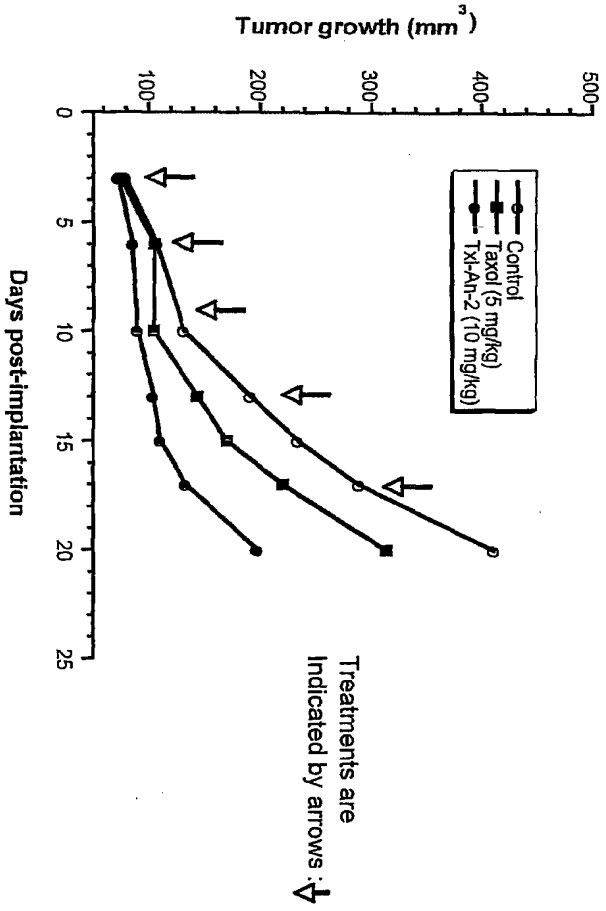

Tumor growth (mm3)
100
200
300
400
500
0
5
10
15
20
25
Days post-implantation
Control
Taxol (5 mg/kg)
Txl-An-2 (10 mg/kg)
Treatments are
Indicated by arrows

Fig. 18A

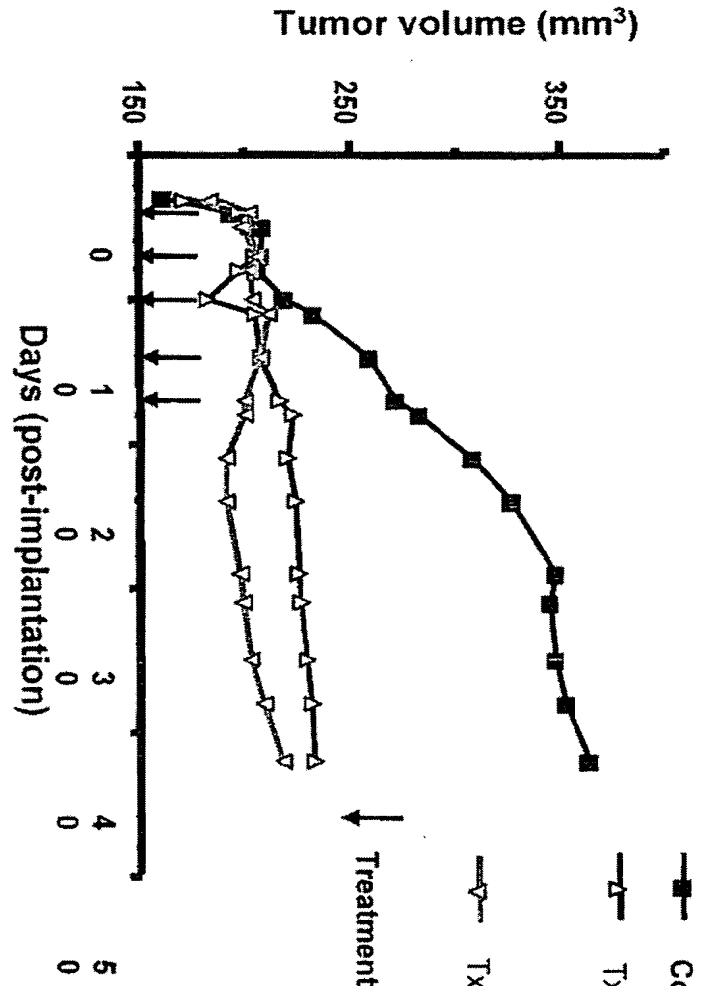
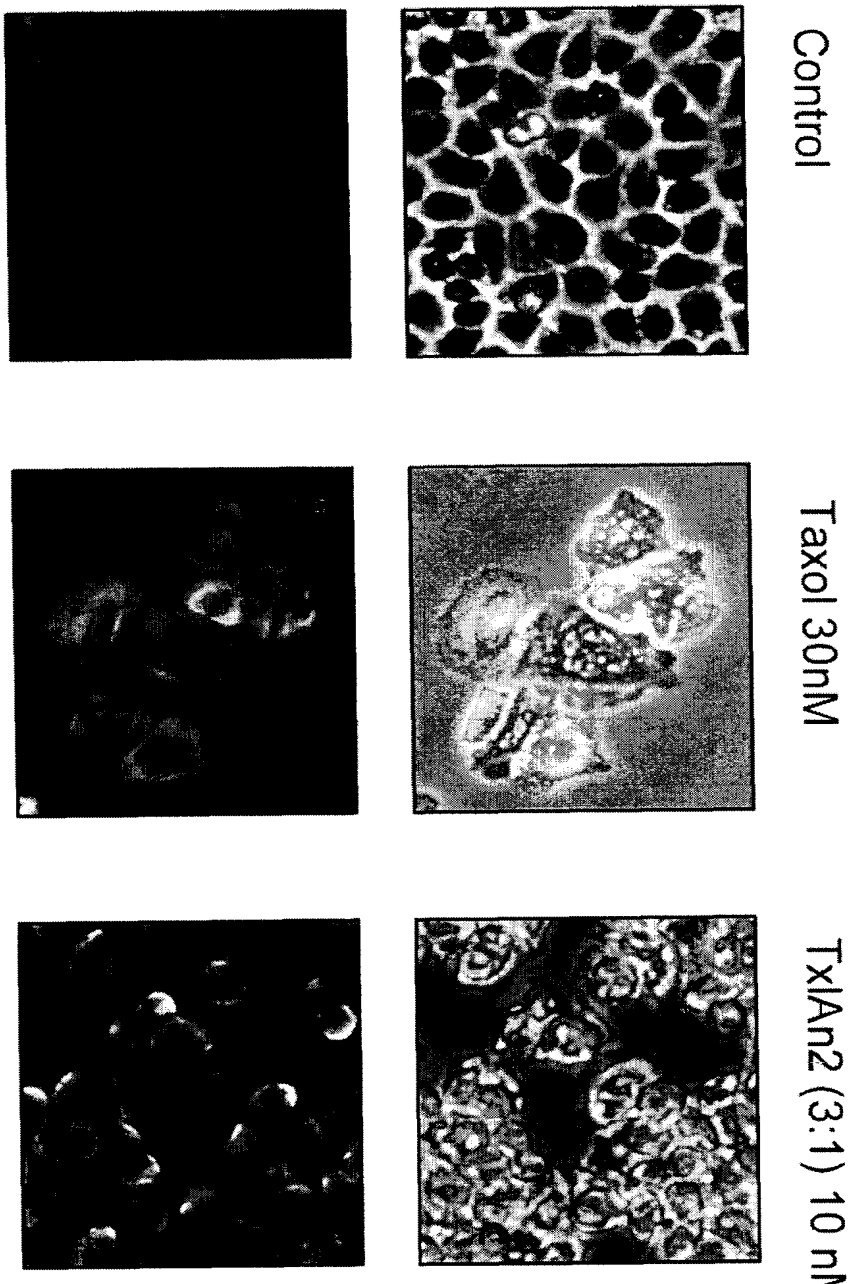

Tumor volume (mm3)
150
250
350
0
100
200
300
400
500
Days (post-implantation)
Control (vehicle) n=5
TxlAn2 (3:1) conjugate fresh (80mg/kg, IP) n=4
TxlAn2 (3:1) conjugate frozen (80 mg/kg, IP) n=5
Treatment on Day 4, 7, 10, 14 and 17

Fig. 18B



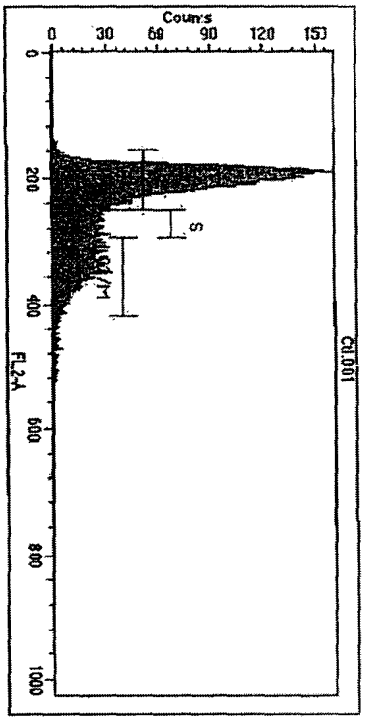
* TxlAn2 (3:1) conjugate equivalent to 30 nM of Taxol (24 hrs treatment)

Fig. 19

Control

File: Ctl.001
Sample ID: Ctl
Patient ID:
Acquisition Date: 03-Apr-06
Gate: G2

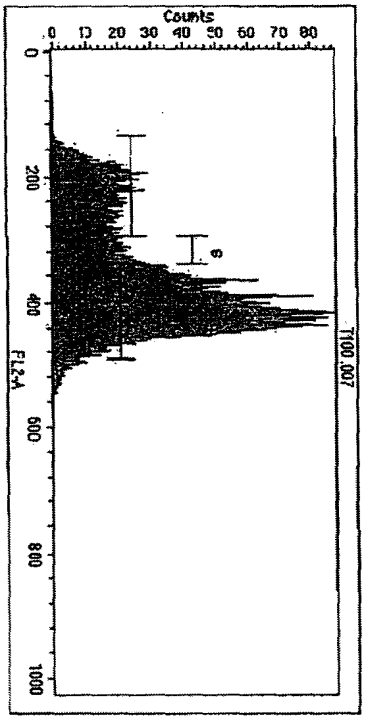
Marker	Events	% Gated
All	9035	100.00
G0/G1	6273	69.43
S	985	10.90
G2/M	1747	19.34



Taxol (100nM)

File: T100.007
Sample ID: T100
Patient ID:
Acquisition Date: 03-Apr-06
Gate: G2

Marker	Events	% Gated
All	9356	100.00
G0/G1	2104	22.49
S	821	8.78
G2/M	6373	68.12



TxlAn2(3:1) (30nM)

File: C30.010
Sample ID: C30
Patient ID:
Acquisition Date: 03-Apr-06
Gate: G2

Marker	Events	% Gated
All	9255	100.00
G0/G1	3379	36.51
S	880	9.51
G2/M	4851	52.41

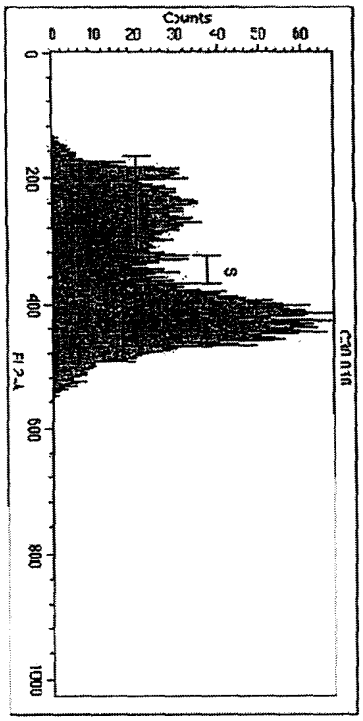


Fig. 20

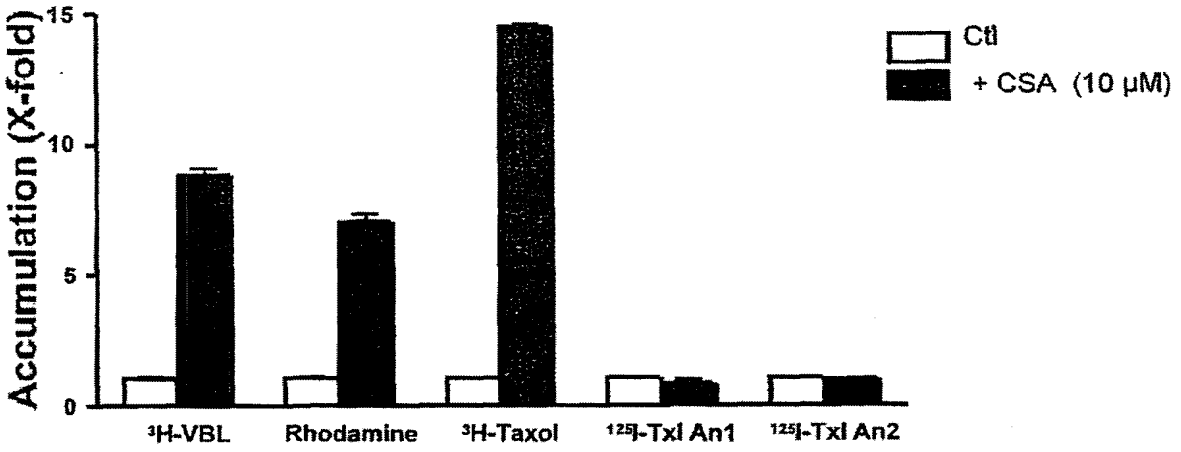


Fig. 21A

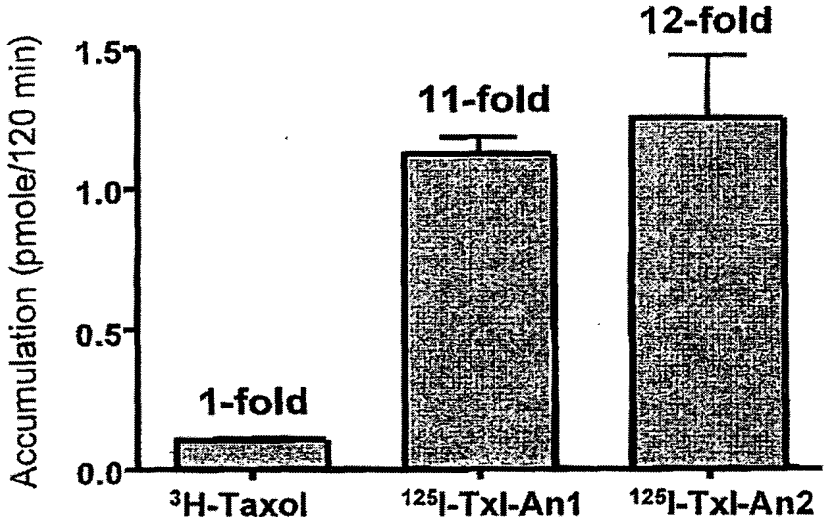


Fig. 21B

LRP expression in brain tumors

A. Primary brain tumors

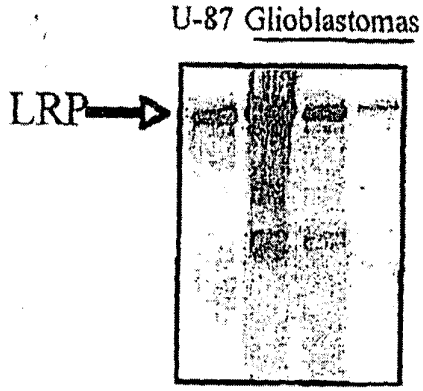


Fig. 22A

B. Brain metastasis from

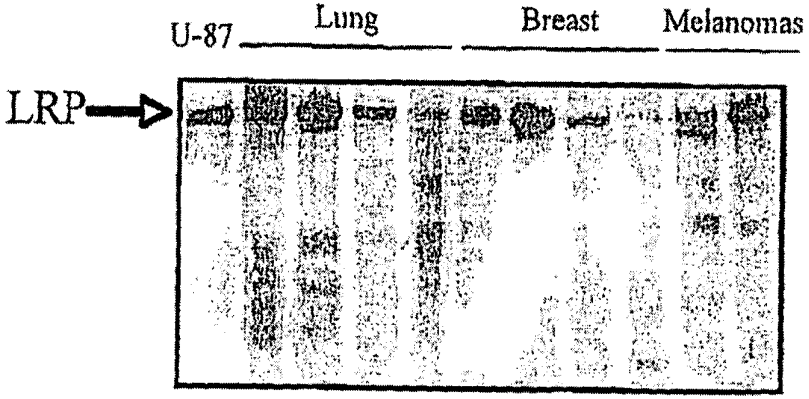


Fig. 22B

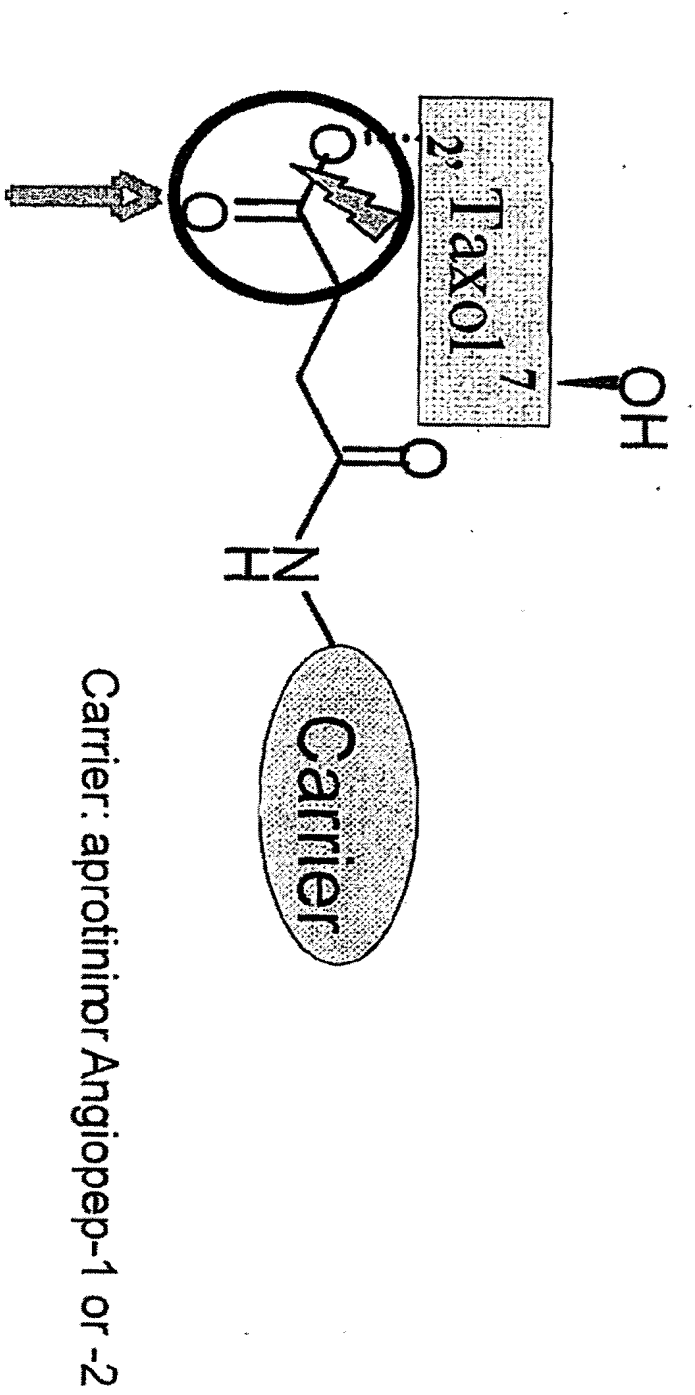

Ester bound cleaved by esterases
2'
Taxol
7
OH
O
O
HN
Carrier
Carrier: aprotinin or Angiopep-1 or -2

Fig. 23A

[TxlAn2 (3:1)]

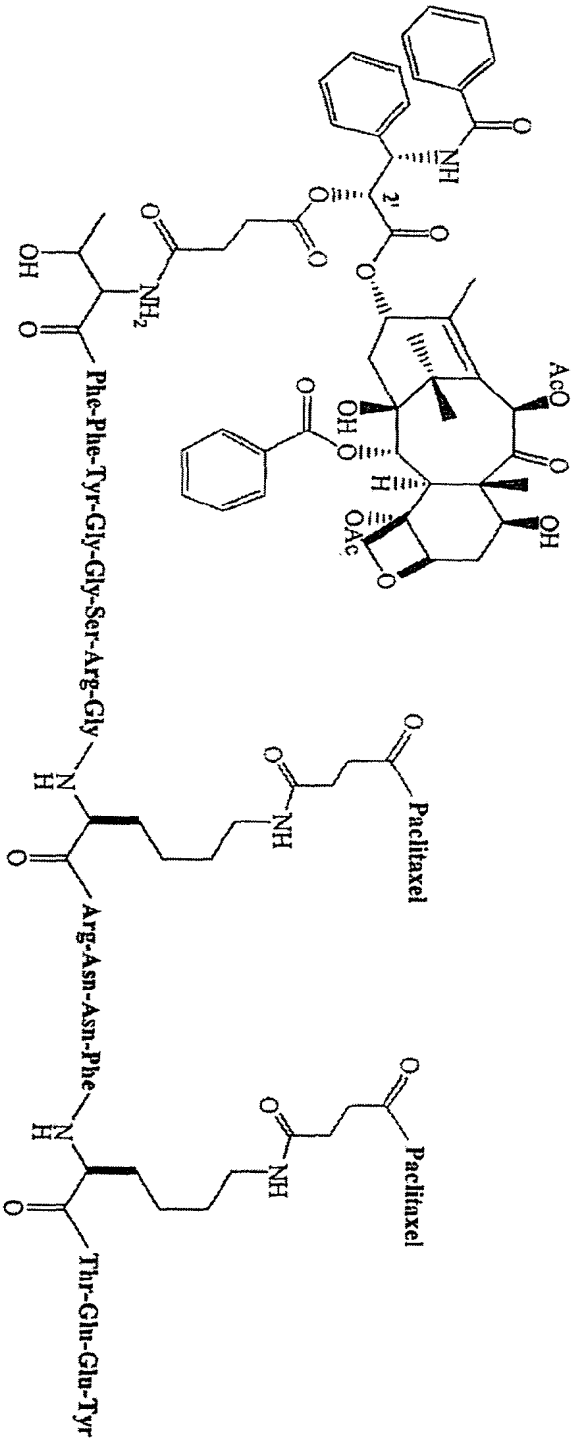


Fig. 23B

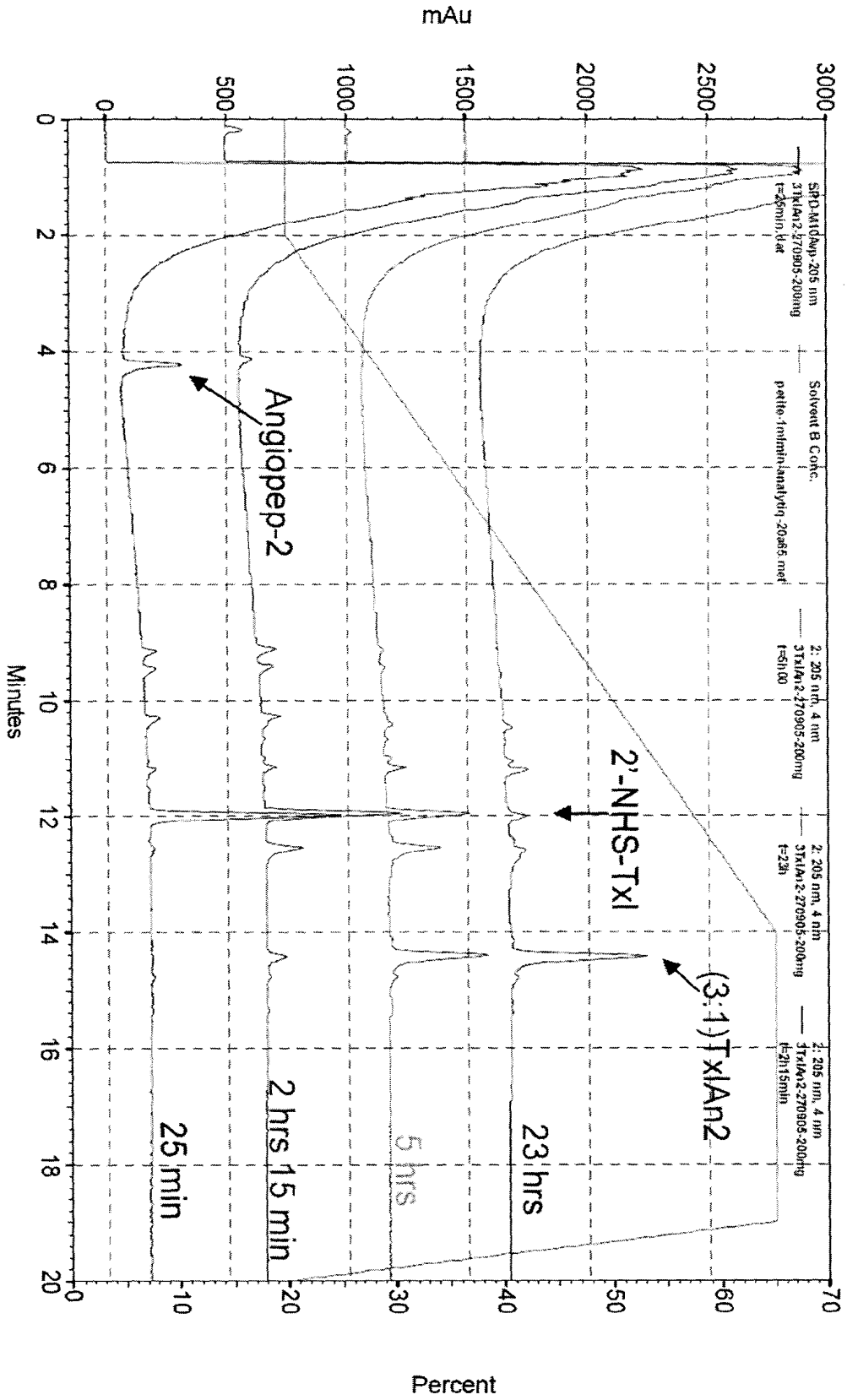

mAu
Minutes
Percent
Angiopep-2
2'-NHS-Txl
(3:1)TxlAn2
23 hrs
5 hrs
2 hrs 15 min
25 min

Fig. 24

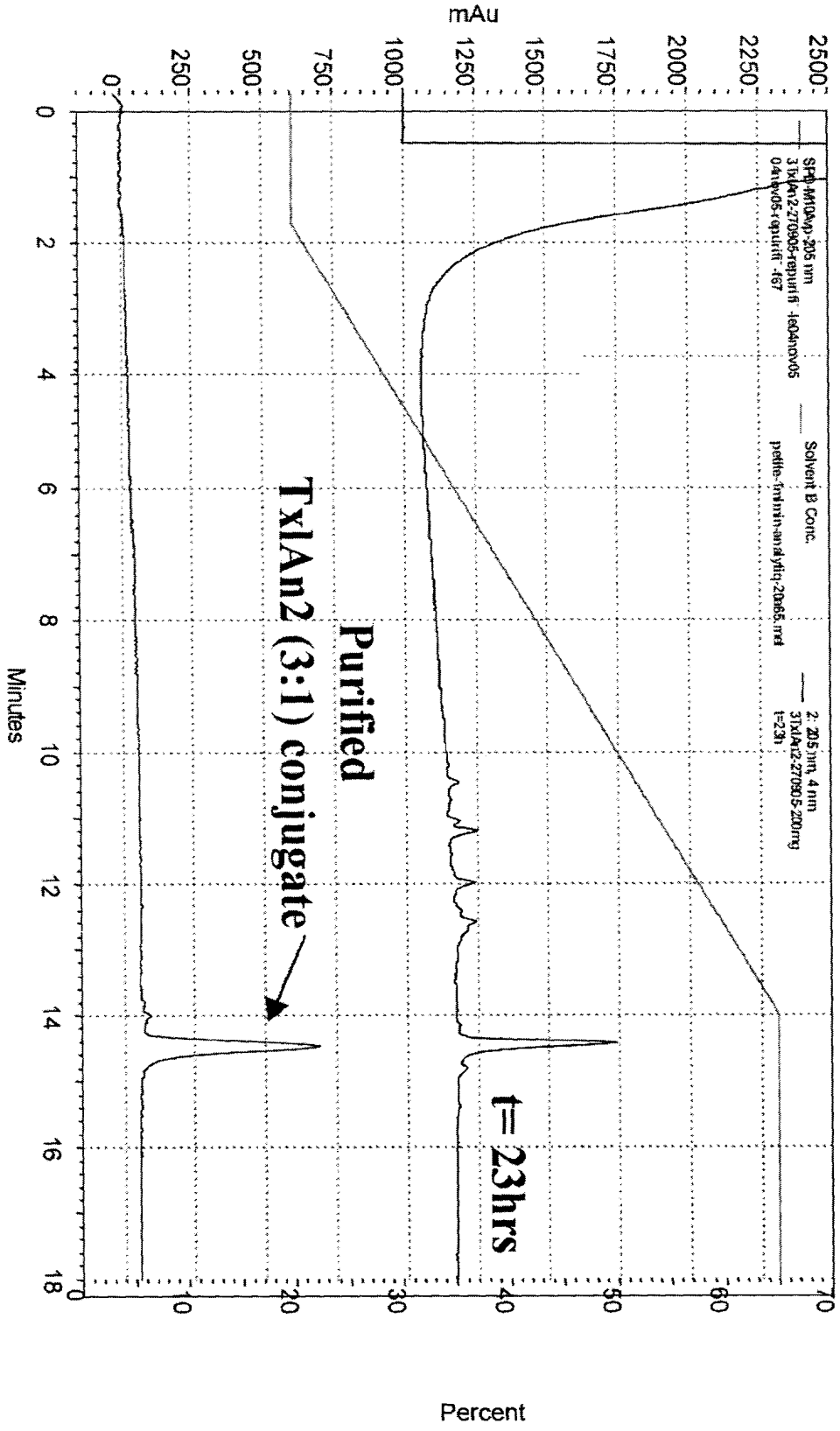


Fig. 25

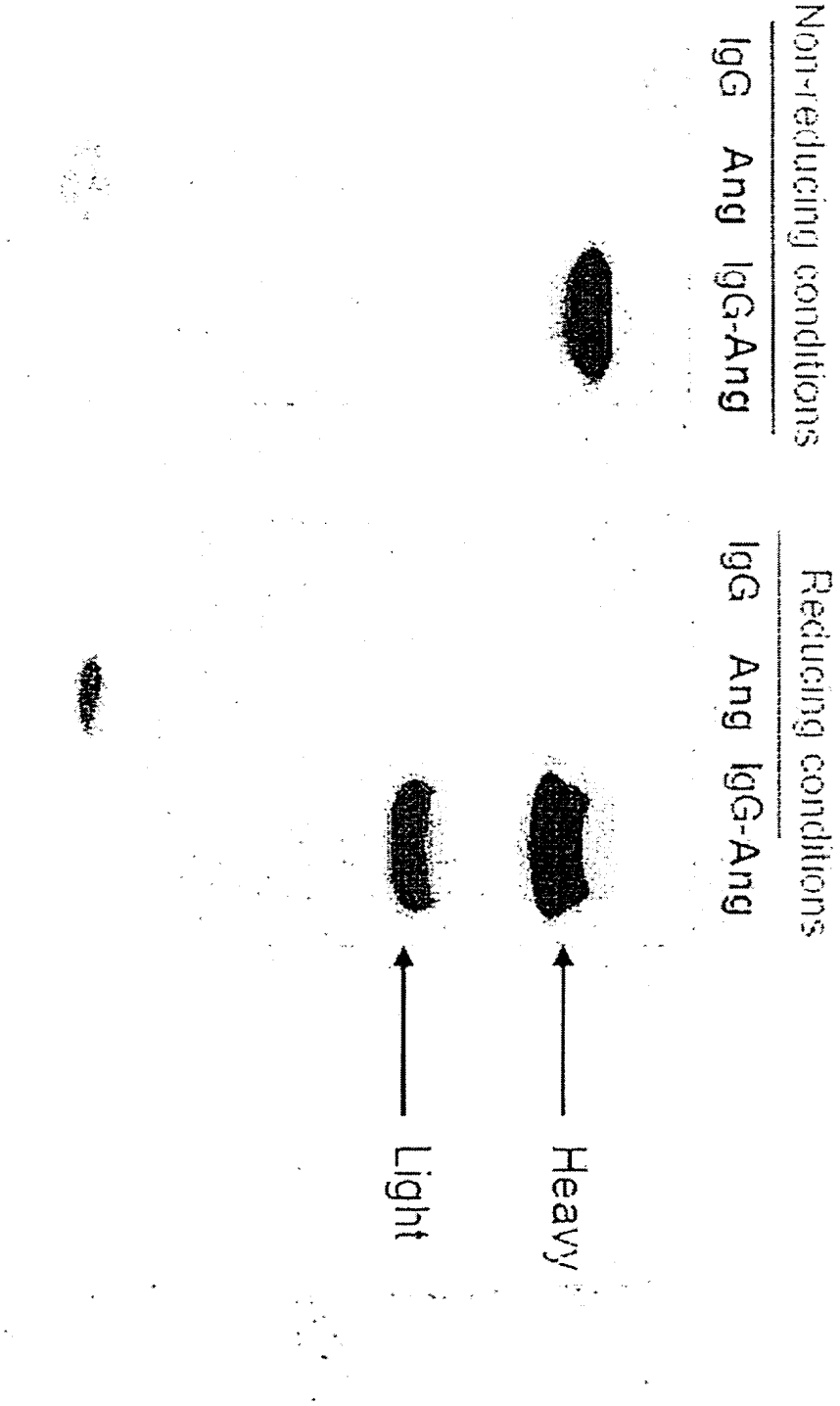

Non-reducing conditions
IgG Ang IgG-Ang
Reducing conditions
IgG Ang IgG-Ang
Heavy
Light

Fig. 26

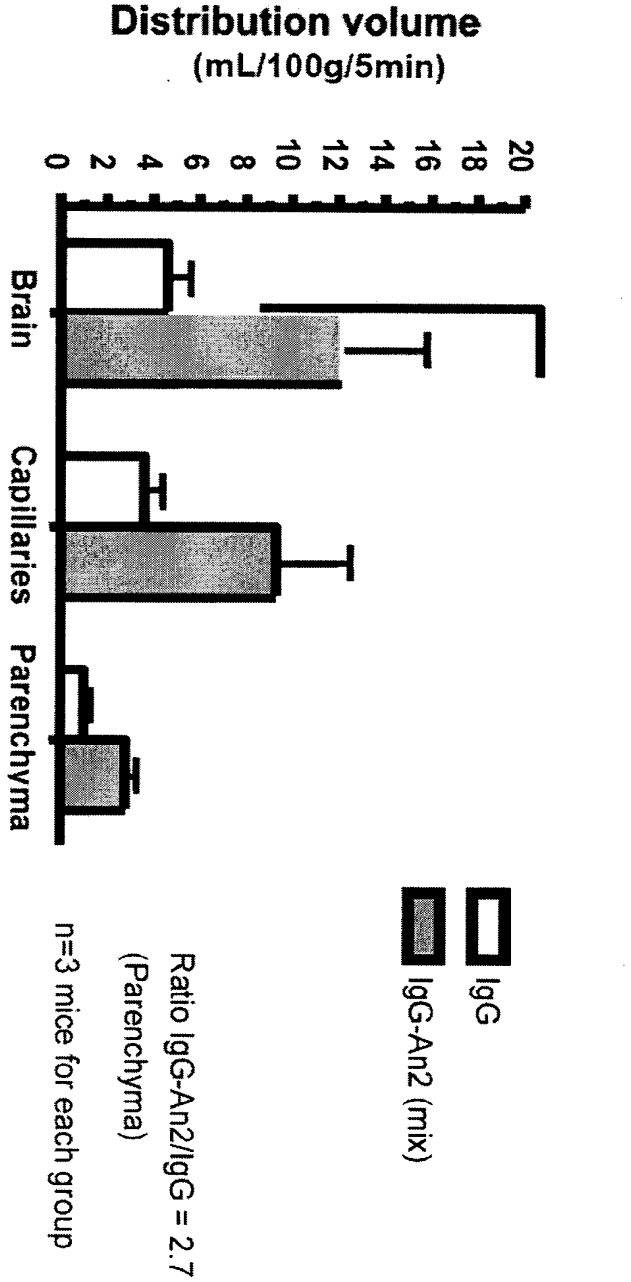

Distribution volume
(mL/100g/5min)
0
2
4
6
8
10
12
14
16
18
20
Brain
Capillaries
Parenchyma
IgG
IgG-An2 (mix)
Ratio IgG-An2/IgG = 2.7
(Parenchyma)
n=3 mice for each group

Fig. 27

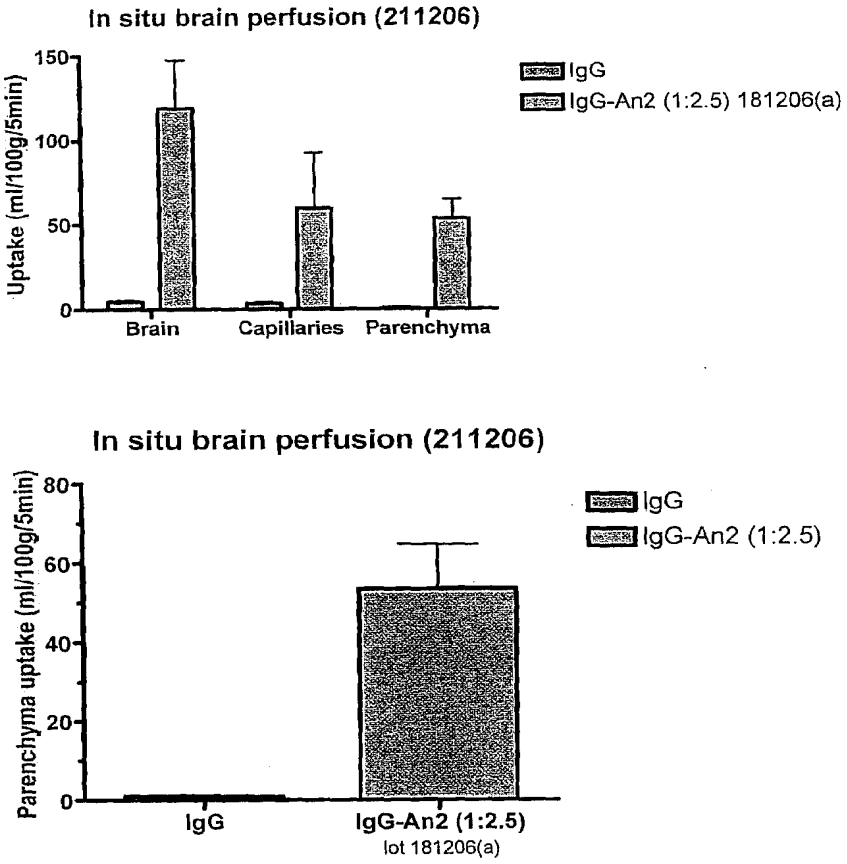

In situ brain perfusion (211206)
Uptake (ml/100g/5min)
150
100
50
0
Brain
Capillaries
Parenchyma
IgG
IgG-An2 (1:2.5) 181206(a)
In situ brain perfusion (211206)
Parenchyma uptake (ml/100g/5min)
80
60
40
20
0
IgG
IgG-An2 (1:2.5)
lot 181206(a)
IgG
IgG-An2 (1:2.5)

Fig.28

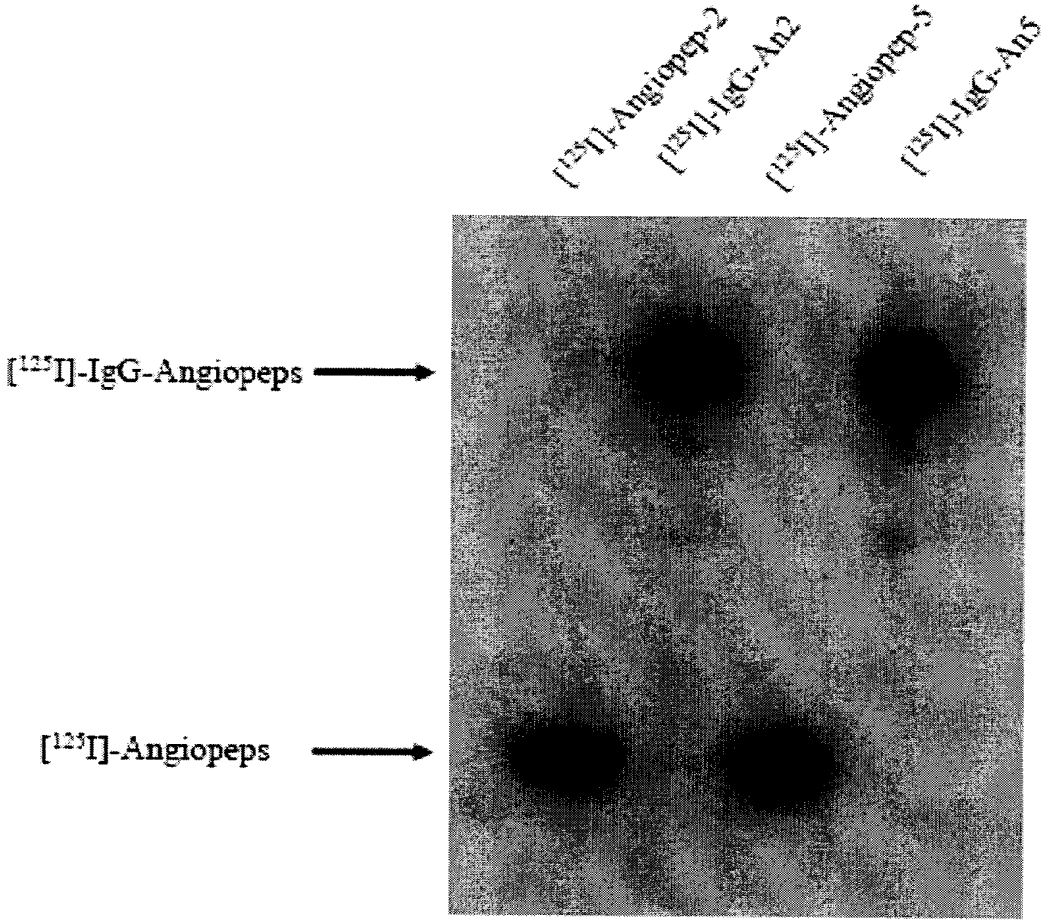

[125I]-Angiopep-2
[125I]-IgG-An2
[125I]-Angiopep-5
[125I]-IgG-An5
[125I]-IgG-Angiopeps
[125I]-Angiopeps

Fig. 29

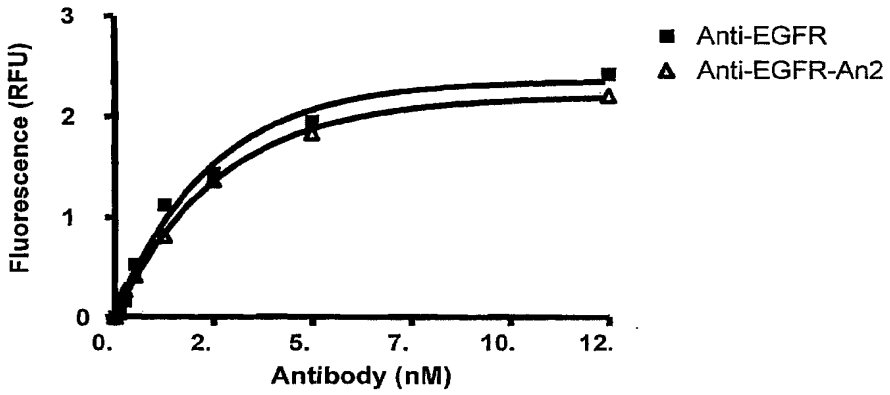

Fluorescence (RFU)
3
2
1
0
0.
2.
5.
7.
10.
12.
Antibody (nM)
Anti-EGFR
Anti-EGFR-An2

Fig.30

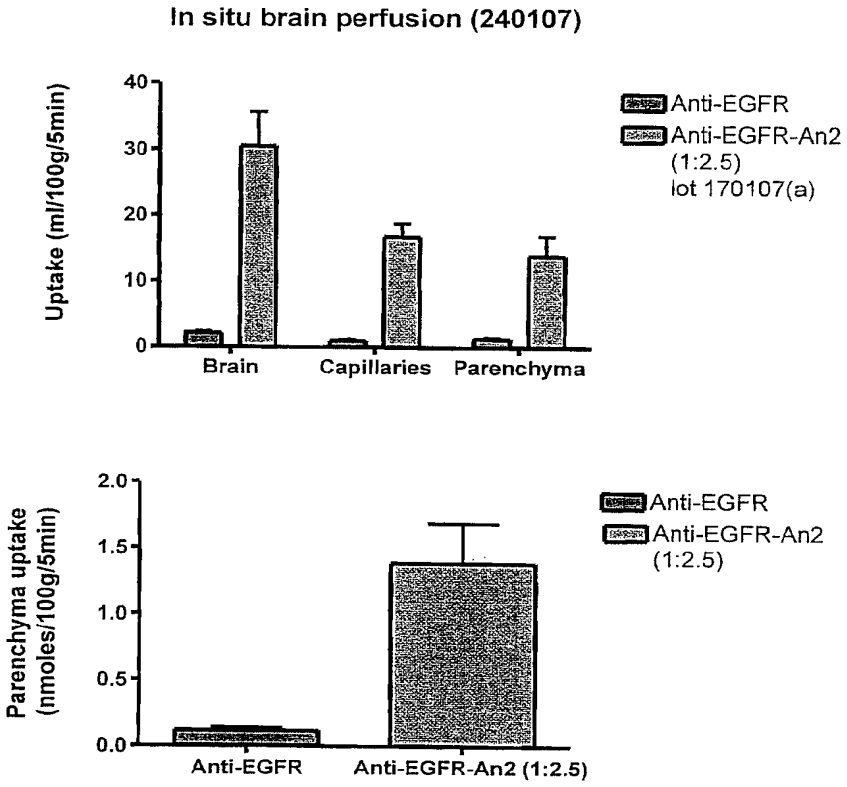

In situ brain perfusion (240107)
Uptake (ml/100g/5min)
40
30
20
10
0
Brain
Capillaries
Parenchyma
Anti-EGFR
Anti-EGFR-An2 (1:2.5) lot 170107(a)
Parenchyma uptake (nmoles/100g/5min)
2.0
1.5
1.0
0.5
0.0
Anti-EGFR
Anti-EGFR-An2 (1:2.5)
Anti-EGFR
Anti-EGFR-An2 (1:2.5)

Fig.31

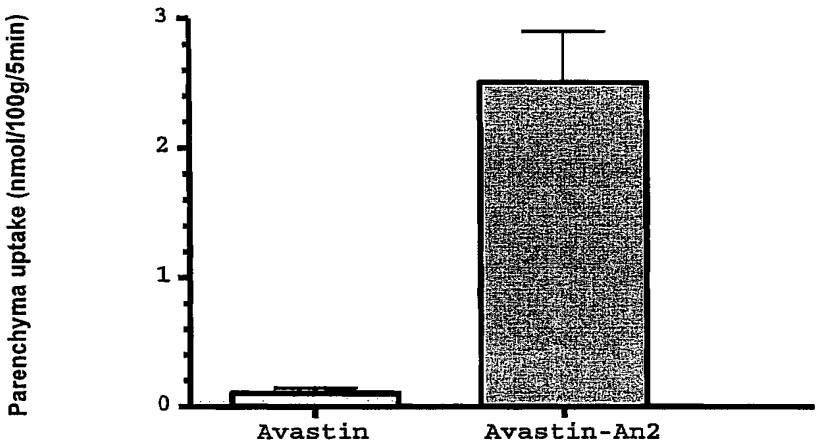


Fig.32

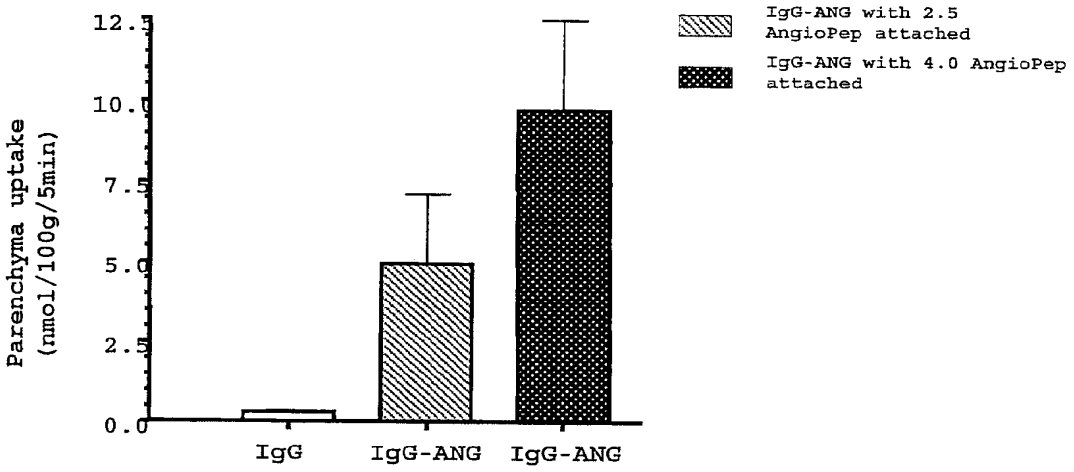

Parenchyma uptake
(nmol/100g/5min)
12.5
10.0
7.5
5.0
2.5
0.0
IgG
IgG-ANG
IgG-ANG
IgG-ANG with 2.5 AngioPep attached
IgG-ANG with 4.0 AngioPep attached

Fig.33

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2008/001030

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C07K 14/81*** (2006.01), ***A61K 31/337*** (2006.01), ***A61K 38/10*** (2006.01), ***A61K 39/395*** (2006.01), ***A61K 47/48*** (2006.01), ***A61P 35/00*** (2006.01), ***C07K 14/47*** (2006.01), ***C07K 7/06*** (2006.01), ***C07K 7/08*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: ***C07K 14/81*** (2006.01), ***A61K 31/337*** (2006.01), ***A61K 38/10*** (2006.01), ***A61K 39/395*** (2006.01), ***A61K 47/48*** (2006.01), ***A61P 35/00*** (2006.01), ***C07K 14/47*** (2006.01), ***C07K 7/06*** (2006.01), ***C07K 7/08*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
Databases: GenomeQuest, GenBank, PubMed, Delphion, Scopus, CAPlus; Keywords: aprotinin, aprotinin-like (poly)peptide, aprotinin-derived polypeptide, blood brain barrier, conjugate, angiopep (in various combininations) and inventors names.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/009229 A1 (BELIVEAU, R. et al) 25 January 2007 (25-01-2007)	1-54
X	WO 2006/086870 A1 (BELIVEAU, R. et al) 24 August 2006 (24-08-2006)	1-54
X	WO 2004/060403 A2 (BÉLIVEAU, R. et al) 22 July 2004 (22-07-2004)	1-3, 5-11, 16-23, 25-31, 36-54

[ ] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 August 2008 (06-08-2008)	15 September 2008 (15-09-2008)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Riad Qanbar 819- 934-7937

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:

   a. type of material

      [X] a sequence listing

      [ ] table(s) related to the sequence listing

   b. format of material

      [X] on paper

      [X] in electronic form

   c. time of filing/furnishing

      [ ] contained in the international application as filed.

      [ ] filed together with the international application in electronic form

      [X] furnished subsequently to this Authority for the purposes of search.

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments :

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. : 46-54

because they relate to subject matter not required to be searched by this Authority, namely :

Claims 46-54 are directed to a method for treatment of the human or animal body by surgery or therapy, which is subject matter the International Search Authority is not required to search under Rule 39.1(iv) of the PCT. However, this Authority has carried out a search based on the alleged effects or purposes/uses of the product defined in claims 1 and 21.

2. [ ] Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

The claims are directed to a plurality of inventive concepts as follows:

Group 1 - Claims 1, 3-12, 14, 16, 17, 19-21, 23-32, 34, 36-54 (all partially) are directed to a polypeptide named angiopep-4b, which has the sequence set forth in SEQ ID NO: 109, conjugates of this polypeptide, and uses of said conjugate.

Continued in Supplemental Box

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2008/001030

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO 2007009229A1	25-01-2007	AU 2006272405A1	25-01-2007
		CA 2614687A1	25-01-2007
		EP 1907009A1	09-04-2008
WO 2006086870A1	24-08-2006	AU 2005327497A1	24-08-2006
		CA 2597958A1	24-08-2006
		CN 101160403A	09-04-2008
		EP 1859041A1	28-11-2007
		US 2006189515A1	24-08-2006
WO 2004060403A2	22-07-2004	AU 2003296232A1	29-07-2004
		AU 2004203682A1	22-07-2004
		BR 0406647A	06-12-2005
		CA 2516056A1	22-07-2004
		CN 1244375C	08-03-2006
		CN 1515314A	28-07-2004
		CN 1747747A	15-03-2006
		EP 1583562A2	12-10-2005
		JP 2006515363T	25-05-2006
		MX PA05007322A	17-02-2006
		US 2006182684A1	17-08-2006
		US 2006189521A1	24-08-2006
		WO 2004060403A3	18-11-2004
		WO 2004060914A1	22-07-2004

Continuation of Box No. 3 III (Observations where unity of invention is lacking)

Group 2 - Claims 1, 3-12, 14, 16, 17, 19-21, 23-32, 34, 36-54 (all partially) are directed to a polypeptide named angiopep-5, which has the sequence set forth in SEQ ID NO: 110, conjugates of this polypeptide, and uses for said conjugate;
Group 3 - Claims 1, 3-12, 14, 16, 17, 19-21, 23-32, 34, 36-54 (all partially) are directed to a polypeptide named angiopep-6, which has the sequence set forth in SEQ ID NO: 111, conjugates of this polypeptide, and uses for said conjugate; and
Group 4 - Claims 1, 3-12, 14, 16, 17, 19-21, 23-32, 34, 36-54 (partially) and 2, 13, 15, 18, 22, 33, 35 (completely) are directed to a polypeptide named angiopep-7, which has the sequence set forth in SEQ ID NO: 111, conjugates of this polypeptide, and uses for said conjugate.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

Groups 1 to 4 feature 19 amino acid aprotinin-like peptides that tend to accumulate in specific body organs. However, an *a posteriori* analysis has revealed that such peptides are known in the art, as exemplified by Beliveau et al, WO 2007/009229, published on 25-01-2007, and cited in the application. Therefore, Groups 1 to 4 are not linked together by a novel and inventive technical feature so as to form a single alleged invention as required by Rule 13.1 of the PCT.